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Genetic diversity, Variation in essential oil content and antibacterial activity among different populations of Juniperus phoeniceaL. from Jordan

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DEDICATION

This endeavor is dedicated to all members of my family
Especially,
my husband Osama AL-Hassanat, and sweet son Bra'a.
To my father, mother, younger brothers and sister,
for their huge sacrifice, moral support, cooperation,
Encouragement, patience, and prayers for my
Health and success
which enabled me to achieve this goal.

Enas Mohammad AL-Khalifeh

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LIST OF ABBREVIATIONS

J. phoenicea	Juniperus phoenicea L.
PCR	Polymerase chain reaction
ISSR	Inter Simple Sequence Repeats
GC-MS	gas chromatography and mass spectrometry
CFU	Colony forming unit
ANOVA	Analysis of variance

ABSTRACT

Genetic diversity, Variation in essential oil content and antibacterial activity among different populations of Juniperus phoeniceaL. from Jordan

Enas Mohammad Al-Khalifeh

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Juniperus phoenicea L. tree (Araâr) is a wild species and characterized by a medicinal value. *Juniperus phoenicea* is found predominantly in the southern heights of Jordan. Due to the threat that may minimize this 3species biodiversity, recently there has been considerable attention paid in understanding its importance and how biodiversity supports ecosystem processes. Thus the genetic and chemical profiles were very much taken into consideration during characterization the biodiversity statues of the *J. phoenicea*.

In the present study, *J. phoenicea* samples from five natural populations in Southern Jordan were collected and their genetic variations were studied. In addition, the hydro-distilled essential oils from three geographical regions were characterized for its chemical composition and antibacterial activity. The genetic variation among and within the selected populations was evaluated by mean of inter simple sequence repeats (ISSR). The analysis of the ISSR data for the two groups showed that a small but significant amount of genetic variations (0.47% of the total) and larger; significant amount (5.3% of the total) are due to differences between regions and variations between individuals within populations, respectively.

GC and GC-MS analysis of the chemical constituents of the isolated oils mainly revealed the presence of monoterpene hydrocarbons, sesquiterpene hydrocarbons, oxygenated monoterpenes, and oxygenated sesquiterpenes. Monoterpene hydrocarbons represent the major constituent in most studied samples among of which α-pinene is the main compound. This study revealed the occurrence of variation in yield and chemical composition of the studied populations according to their geographical origin, extracted part and genetic influence. The essential oils exhibited wide range of antibacterial activities (weak to excellent) as measured by hole plate diffusion and MIC assay. A correlation between the variation in chemical composition and antibacterial activity of the principal oils was observed, and the recorded variation in the antibacterial activity found to be significant.

الملخص

التنوع الوراثي الكيميائي لنبات العرعر الفينيقي في الاردن وفعالية الزيوت المستخلصة منه ضد بعض الجراثيم

ايناس الخلايفة

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يعتبر العرعر الفينيقي (Juniperus phoenicea L.) واحدا مـن اصـناف النباتـات الطبية البرية المعمرة، ذات الفائدة العلاجية الكبيرة والتي تعزى بـشكل اساسـي لمحتواهـا الوفير من الزيوت الطيارة. في الاردن ، يمثل العرعر الفينيقي الجزء الاكبر مـن الغطـاء الخضري لمرتفعات جبال الشراه الجنوبية والتي كانت تزدهر كثيرا بهذا النبات. ولكن هـذا الغطاء تقلص كثيرا خلال القرن الماضي بسبب عدة عوامل. وعليـه فـان دراسـة التنـوع الحيوي لهذا النبات بناءا على تنوعه الوراثي والكيميائي تعد ذات اهمية كبيرة. فـي هـذه الدراسة، تم اختبار التغاير الوراثي بين عينات العرعر الفينيقي التي تنتمـي الـى خمـس مجموعات طبيعية مختلفة في جنـوب الاردن باسـتخدام تقنيـة Inter Simple Sequence Repeats (ISSR). اظهرت سلسة تفاعلات البوليمرز (PCR) للمـادة الوراثيـة للعينـات المدروسة وجود تغاير وراثي بين العينات في نفس المجموعة الواحدة بنسبة (5.30% مـن المجموع الكلي). بينما مثل التغاير الوراثي بين المجموعات المختلفة نسبة اقل (0.47%مـن المجموع الكلي). استخدمت الزيوت الطيارة المستخرجة بواسطة التقطير من اوراق وتـوت نبات العرعر الفينيقي منفصلة و جمعت من ثلاث مناطق جغرافية مختلفـة فـي دراسـة التنوع الكيميائي . اظهرت نتائج التحليل الكروماتوغرافي (GC and GC-MS) ان زيـوت العرعرالفينيقي تتكـون بـشكل رئيـسي مـن مركبـات التربينـات : (monoterpene hydrocarbons, sesquiterpene hydrocarbons, oxygenated monoterpenes, and oxygenated sesquiterpenes.). حيـث كانـت التربينـات الاحاديـة (monoterpene hydrocarbons) هي الاكثر في جميع الزيوت المدروسة باستثناء الزيت من عينة الدراسـة الماخوذه من منطقه وادي رم . بخلاف العينات الاخـرى كانـت مركبـات oxygenated

sesquiterpenes هي المكون الرئيسي في عينة الدراسة الماخوذه من منطقـه وادي رم .
لوحظ وجود تغاير كمي بين مكونات الزيوت المدروسة وذلك حـسب تنوعهـا الجغرافـي
وحسب الجزء المستخدم لاستخلاص الزيت. وايضا حسب تغايرها الوراثي . تم اختبار فعـا
لية الزيوت المستخرجة من اوراق وثوت العرعر الفينيقي كل على حدا ضد اربعة انوع مـن
البكتيريا الممرضه(**Escherichia coli and Bacillus cereus, Staphylococcus aureus ,**
Pseudomonas aeruginosa). اظهرت النتائج الاولية فعالية معظم الزيوت المـستخرجة
ضد جميع انواع البكتيريا التي تم اختبارها.كما اظهرت النتائج وجود تغاير الفعالية بينهـا.
كان التغاير له علاقة مباشرة بمحتوى الزيوت الطيارة. وذلك حـسب تنوعهـا الجغرافـي
وحسب الجزء المستخدم لاستخلاص الزيت .

CHAPTER ONE
Introduction

1.1. Medicinal plants

There is no specific time in history mention when did human early discovered the treasure of medicinal plants but surly it keeps remind us about how much these types of plants and their extracts long were with great value as food and cure.

Vast amount of scientific evidence is available now demonstrating broad range of pharmacological and nutraceutical activities of medicinal plants (Burt, 2004; Celiktas et al., 2007; Edris, 2007). These include antimicrobial (Hammer et al., 1999; Hussain et al., 2009; Obeidat, 2011), antioxidant (Jayaprakasha et al., 2006; Paradiso et al.,2008; Descalzo and Sancho 2008), anticancer (Kumar et al., 2004; Sylvestre et al., 2006; El-Sawi, and Motawae, 2008; Peres et al., 2009; Biswas et al., 2010; Mothana et al., 2009) and anti-inflammatory activities (Mukherjee et al., 2000 ;Ozturk et al., 2002).

Nowadays public attitudes towards natural products which obtained from medicinal plants have been raised. This in turn led to increase the demands on them. Besides there traditional usage in folk medicine, Medicinal plants have multiple commercial applications such as the pharmaceutical, agronomic, cosmetics perfumes and soft food industries (Sivropoulou et al., 1996; Buchbauer, 2000; Burt, 2004; Delamare et al., 2007).

Large demand on medicinal plants is contributing to the loss of its biodiversity (Phillipson, 1997). In addition, Medicinal plants as part from plant communities are threatened by many factors such as; climate change, overexploitation, Human Disturbance, invasive species and pollution (IUCN, 2008; CBD, 2008; Isbell et al., 2011), Consequently large number of them become rare or faces the threat of extinction among of which Juniperus phoenicea species.

J. phoenicea is a wild plant species that characterized by a remarkable medicinal value. It is the only Juniperus species found in Jordan exclusively in the southern heights and on altitudes exceeding 1000 m (GCPE, 2001) and it is locally known Araâr (Figure 1.2, 1.3 and 1.4).

The characterization of J. phoenicea statues based on its genetic and chemical profiles is more highlighted due to the threat which may lead to declining its diversity statues in natural habitat of Jordan.

1.2. Genetic Variation

Genetic diversity between populations of species affects their physical characteristics, viability, productivity, resilience to stress and adaptability to

change (Global Biodiversity Strategy, 1992). Understanding and proper assessment of genetic diversity among and within living populations of plants is prerequisite to evaluate its availability status, therefore design suitable management strategies for its conservation (jordu et al., 2001; Lockwood et al., 2007).

Fortunately, progress in biotechnology approaches has offered different marker systems for diagnosis of genetic diversity. Practically, (PCR) technology has led to the development of many simple and quick techniques for DNA fingerprinting. These include amplified fragment length polymorphisms (AFLP), sequence tagged site (STS) which based on primers derived from restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) which detects nucleotide sequence polymorphisms using a single primer of arbitrary nucleotide sequence, and Inter Simple Sequence Repeats (ISSR) which permits detection of polymorphisms in inter-microsatellite loci, using a primer designed from dinucleotide or trinucleotide simple sequence repeats (Zietkiewicz et al., 1994).

(ISSR) has only recently been developed as RAPDs-like approach that assesses variation in the numerous microsatellite regions dispersed throughout the various genomes. this technique involves the polymerase chain reaction (PCR) amplification of regions between adjacent, inversely oriented microsatellites, using a single simple sequence repeat (SSR) motifs (di-, tri-, tetra-, or pentanucleotides) containing primers anchored at the 3' or 5' end by two to four arbitrary, often degenerate nucleotides (Depeiges et al., 1995).

Extensive previous work with different junipers species has found that dominant marker like ISSRs and RAPD analyses can be used to distinguish among major taxonomic sections and varieties (Adams and Demeke, 1993), to place uncertain specimens into species, to support the recognition of new species and varieties (Adams and Turuspekov, 1998), to group species, and to differentiate and group populations of a species (Adams et al., 1993). However ISSRs have advantages over many classes of available molecular markers because they do not require preliminary sequence information, they are less prone to laboratory conditions than RAPDs. Moreover, it has been successfully used to estimate genetic variation for Juniperus phoenicea (Adams et al., 2003; Meloni et al., 2006).

1.3. Essential Oils

The soul of healing properties of medicinal plants, e.g., anti microbial properties, is mainly due to its essential oil content (Edris, 2007). The powerful therapeutic value of essential oils has been known for centuries. For

many civilizations it were more valuable than gold. Now, we only rediscover it.

These essential oils are complex mixtures of organic compounds (Gouges et al., 2005). They are generally liquid, aromatic and possess pleasant odor and essence (Burt, 2004). They are generally identified with the name of the source plant.

Organic compounds constituents in the essential oils are generally comprised terpenes and aromatic compounds (Dudareva et al., 2006). The terpenes are the unsaturated hydrocarbons which derived from the simple isoprene molecule which has the molecular formula C_5H_8. The basic molecular formulae of terpenes are multiples of that, $(C_5H_8)_n$. Terpenes can form building blocks by joining together in a "head-to-tail" configuration (CH2==C(CH3)—CH==CH2) to form monoterpene, sesquiterpenes, diterpene and larger sequences (Pinder, 1960). Monoterpenes, Sesquiterpenes, and their oxygenated derivatives comprise about 90- 95% of the essential oil composition (Achak et al., 2008) among of which pinen and its derevatives are the chief compounds comprising J. phoenicea essential oil (Adams et al., 1996; Angioni et al., 2003; Cosentino et al., 2003; El-Sawi et al., 2007; Mazari et al., 2010; Adams et al.,2009; Derwich et al., 2010).

i. Monoterpenes

Limonene **δ-3-Carene** **α-pinene**

α-terpinylacetate **phellandrene**

ii. Sesquiterpen

Cedrol **δ-Cadinene**

Amorphene

Figure 1.1. Chemical structure of some major components of essential oil J. phoenicea

The essential oils can be produced in almost all plant organs such as flowers, buds, stems, leaves, fruits, seeds and roots etc. These are accumulated in secretary cells, cavities, channels, and epidermic cells (Burt, 2004). However essential oils from J. phoenicea are typically extracted from fresh or dried berry like fruit and leaves (IUCN, 2005).

Different methods are employed for isolation of essential oils from their plant source. These include steam distillation, hydrodistillation, liquid carbon dioxide or microwaves, low or high pressure (Bousbia et al., 2009; Donelian et al., 2009). Steam distillation is the preferable choice for obtaining essential oils in food and pharmacological applications (Baker et al., 2000; Kulisic et

al., 2004; Masango, 2004). However hydrodistillation applied successfully for investigation purposes (Guillén et al., 1996; Bilia et al., 2000).

The complexity of the essential oils is a real challenge for determining their accurate compositional data but the rapid advances in spectroscopic and chromatographic techniques have simplified this mission. GC and GC-MS has been proved to be an efficient method for the characterization of essential oils (Bakkali et al., 2008). It's applied successfully on J. phoenicea. The combination of gas chromatography and mass spectrometry (GC-MS) allows rapid and reliable identification of essential oils components (Adlard et al., 2001; Adams, 2007)

Many factors, such as genetic, physiological, environmental and geographical variations affect essential oil yield and its components in medicinal plants (Zobayed et al., 2005; Adams and Nguyen, 2006; Figueiredo et al., 2008). More over chemical polymorphisms have been reported for many medicinal species (Jaime et al., 2000; Pourohit and Vyas, 2004; Curado et al., 2006; Viljoen, 2006; Rahimmalek et al., 2009). Including J. Phoenicea (Adams et al., 1996; Cavaleiro et al., 2001; Rezzi et al., 2001; Adams et al., 2009; Derwich et al., 2010).

1.4. Antimicrobial properties of essential oils

Microbes are rapidly overwhelming today's synthetic drugs as they naturally evolve to avoid antibiotics. The resistance that many pathogens such as viruses and bacteria showed against antibiotics appears to have speeded up in the last decade, largely because antibiotics are being overused giving the germs more chances to mutate (Brock et al, 2012).

Essential oils are very rich in biologically active compounds. Many of which proved to possess multiple activity against microorganisms including: antibacterial (kim et al., 1995; Prabuseenivasan et al., 2006; Hussain et al., 2009), antifungal (Daferera et al., 2000; Sridhar et al., 2003; Mihailovi et al., 2011), antiviral (Tkachenko et al., 2007; Alim et al., 2009; Garozzo et al., 2009). Microorganisms such as viruses and bacteria do not become resistant to essential oils as they do to modern- antibiotics simply because they cannot mutate with it while Pathogenic microorganism can easily mutate and adapt to the drug, making it useless.

Multiple researches conducted into tests of antimicrobial activity have shown that essential oil recovered from J. Phoenicea demonstrated antimicrobial activity against different pathogens (Angioni et al.,2003; Cosentino et al., 2003; Derwich et al., 2010; El-Sawi et al., 2007 and Mazari et al., 2010).

1.5. Statement of the problem

Jordan is a relatively small country situated in the heart of Middle East. Despite of the small land area it combines wide climatic circumstances and variety of geographical patterns. For this it has a unique plant biodiversity composition consisting of more 2500 vascular plant species, among them 485 species from 99 different families are classified as medicinal plants (Al-Eisawi, 1982; Oran and Al-Eisawi, 1998; Afifi and Abu-Irmaileh, 2000) but unfortunately a lot of them are considered rare or endangered (CBD, 2009).

The Southern area of Jordan was rich in vegetation cover more than now. Wide areas particularly the mountainous heights were covered with J. phoenicea and Cypress forests, but now it become some scattered forest remnants (GCPE, 2001). Studies indicate that woodlands in the southern region of the country have historically suffered from strong impacts represented by transforming forest lands into other land uses, continuous over-grazing, hard environmental conditions, long excessive exploitation of natural resources and movement of armies invading the region or passing through it which led to significant forest fragmentation specially those of J. Phoenicia (GCPE, 2001; CBD, 2009).

J. phoenicea is one endangered wild plant species in Jordan that characterized by important medicinal value. It is the only Juniperus species found In Jordan exclusively in the southern heights and on altitudes exceeding 1000 m and it is locally known Araâr (Figure 1.2, 1.3 and 1.4).

In 1998 J. phoenicea was listed on IUCN Red List of Threatened Species (IUCN, 1998). The deterioration in J. phoenicea forests may lead to affect its genetic diversity. However a few studies related to the evaluation of the diversity statues of most, rare and endangered flora like J. phoenicea have been carried out in Jordan (CBD, 2009).

For this the current study has the following Aims and Objectives:

i. Assess the genetic variation statues among and within five natural populations of J. phoenicea from the following areas of S.Jordan: Al-Shoubak, Al-tybeh, Wadi-Musa, Dana and Wadi Rum using ISSR molecular technique.

ii. Exploration of The yield and detailed chemical profile of the selected populations using state-of-the-art chromatographic techniques.

iii. Investigate the chemical variation among the studied populations with regards to their geographical origin, part used for extraction, genetic and environmental influence.

iv. Evaluate the bio activity of J. phoenicea essential oil of selected samples on inhibiting the growth of some pathogenic bacteria and compare this with some commercial drugs.



Figure 1.2. J. phoenicea tree, Hewalah region Al-shoubak 14-5-2011

Figure 1.3. J.phoenicea shrubs, Little Petra, 29-4-2011



Figure 1.4 Branch of J.phoenicea tree, red-brown berry (cone) and needles like leaves are appear, Al-shoubak, 14-5-2011

CHAPTER TWO
Review of literature

2.1. Description of Juniperus phoenicea L. Tree

2.1.1. Taxonomy and Ecology

Juniperus phoenicea L. specie belongs to the genera Juniperus which includes nearly 70 species (Dirr, 1983; Adams, 1996; Adams, 2004; Adams, 2009). Temperate and subtropical regions of the northern hemisphere is the home land of these species, also its distribution goes further to south of the equator in Africa with only one species was found (Gaussen, 1968; Dirr, 1983; Adams, 2004). It goes from the upper arid to the humid in the hot and mild variants (Gaussen, 1968). The minimal annual rainfall must be about 150 mm (IUCN, 2005).

The detailed taxonomy of J. phoenicea is listed in Table 2.1 (Farjon, 1992).

Table 2.1
Taxonomy of Juniperus Phoenicia L.

Kingdom	Phylum	Class	Order	Family	Scientific Name	Species Authotity
PLANTAE	TRACHEOPHYTA	CONIFEROPSIDA	CONIFERALES	CUPRESSACEAE	Juniperus phoenicea	L.

2.1.2. Morphological Description

As described by Zohari (1966), J. phoenicea is horizontal shrub or tree that can live up to 300 years and can be 8 m in high. It has a short trunk, sometimes 2 m. in circumference, and the bark peels off in strips. The leaves have a scaly appearance with pseudo serrated margin (Adams and Demeke, 1993; Adams, 2008) and they are small in 4-6 Overlapping ranks, like those of cypress. Pollination results in the forming of a berry-lfc ike fruit that have a size of a chick-pea and red –brown color with bluish tints. Flowering takes place in winter-spring and the seeds will be ripening following year in summer season (Zohari, 1966).

2.1.3. Geographical Distribution

J. phoenicea species is belonging to the northern hemisphere (Adams and Demeke, 1993). Globally, the distribution area covers the whole Mediterranean basin, that ranging from Portugal to Saudi Arabia, where the species is present mainly with scattered populations in littoral sites. It is also

native to North Africa in Algeria, Morocco and Canary Islands (Gaussen, 1968; Adams and Demeke, 1993).

In Jordan, Phoenician Juniper characterized by extensive but discontinuous distribution which occur exclusively in south west region of the country (CBD, 2009), starting from AL- Tafeilah to Wadi Rum along Al-Sharah mountains.

Jordanian government had always pay much of attention toward plant biodiversity conservation, therefore a number of natural reserves and protected area have been created in different location in the Kingdom, among them: Dana 'Wild Life Natural Reserves' at AL- Tafeilah, Little Petra and Wadi Rum protected areas . Fortunately, these protected areas include parts of Phoenician Juniper forest.

2.1.4. Importance of Juniperus phoenicea Tree

J. phoenicea is a tree with deep roots embedded in history. Records showed that Juniperus phoenicea berries have been found in ancient Egyptian tombs which belong to 240 B.C. (Manniche, 1999).

The tree is known for its effective as well as versatile medicinal applications. Its healing properties are simply wonderful. Essential oils and extract recovered from this tree has been used for centuries as a steam inhalant were it believe to have an effect leans towards the digestive, respiratory and circulatory systems (Bellakhder, 1997).

Some of the common medicinal and Pharmacological uses of J. phoenicea are summarizing in Table 2.1.

The health benefits and the pharmacological action of J.phoenicea are well documented in many bibliographies. For example, the safety and toxicity of J.phoenicea were assessed and reported by Johnson (2001), the cytotoxici activity of the berries from this plant is supported by a few researchers (El-Sawi et al., 2007; El-Sawi and Motawae, 2008), and a strong hypoglycemic effect from both leaves and berries extract has been confirmed by Sanchez de Medina and his group (Sanchez de Medina et al., 1994). Studies conducted by (Ennajar et al. 2009) and (Ali et al. 2010) described the antioxidant and potential protective role of J.phoenicea extract, and the anti diarrheal effects of J. phoenicea leaves extract were also have been confirmed by a group of researchers (Qnais et al., 2005).

Table 2.2.1
Some of the common medicinal uses and their extract parts of J. phoenicea.

Used parts	Common medicinal use	Reference
Leaves	1. To treat Diarrhea and rheumatism	
	2. Clarify bronchitis and Antiseptic for respiratory track	Bellakhder (1997)
	3. Antiseptic activities for the urinary tract	
	4. Diuretic agent	
	5. Treatment for cold and bloated stomach.	
Berries	1. The syrup of sweet ripen berries widely Used to control diabetes	
	2. Help to treat Eczema.	Amer et al. (1994)
Mixture of Leaves and Berries	1. Oral hypoglycemic agent	Amer et al,(1994)
	2. Improve Prostate health	Angioni et al.(2003)
	3. Help increase the flow of digestive fluids,	
	4. Improve digestion and eliminate gas and stomach cramping	Uphof, (1968)

2.2 Genetic Variation

Genotype is typically defined as "the genetic make-up of an organism, as characterized by its physical appearance or phenotype", while genetic variation is the amount of variation regard genotype in a particular population (Global Biodiversity Strategy, 1992).

2.2.1. Using (ISSR) in Genetic Variation study

Recently, Dominant markers, such as RAPDs and ISSRs, have become popular in genetic diversity analysis. These types of markers are especially attractive given their hypervariable nature, the vast numbers of loci that can be examined and the small amount of fresh or dried material used per sample (Adams, 2000; Culley and Wolfe 2000; Sica et al., 2005).

Inter-simple sequence repeat (ISSR) markers were originally used for differentiating among closely related plant cultivars but have become extremely useful for studies of natural populations of plants, fungi, insects, and vertebrates (Wolf, 2005).

Reduced reproduciblity is one major problem facing worker with RAPDs and ISSRs. However the ISSR primers sequence is usually larger, allowing for a higher primer annealing temperature which results in greater band reproducibility than RAPD markers (Wolfe and Liston, 1998). It is prudent to optimize all parameters of the ISSR assay especially DNA quality and concentration, primer and magnesium concentration and cycling conditions (especially annealing temperature). In addition to that using proofreading DNA polymerase considered an excellent choice for ISSR applications

though it have a high-performance features and very low probability of base mis-incorporation.

2.2.2. Genetic Variation of J. phoenicea

Juniperus is very divers genera (Demeke and Adams, 1994), comprises nearly 70 species with 26 varieties (Adams, 1999). Based on DNA fingerprint and morphological observations the species of J. phoenicea was clearly divided into only two variance (Taxa): var. phoenicea with round shape or globose female cone (berries) and var. turbinate with turbinate (= elongated) shape or obovoid female cone (Gaussen, 1968; Adams, 2002).

Both taxa were extensively studied based on morphological observations, DNA fingerprint and leaf terpenoids that's was for different countries including Greece, Spain, Portugal, Morocco and Canary island (Adams, 1996; Cavaleiro et al. 1996; Adams, 2002)

Further DNA evidences showed the presence of variations in genetic profiles at the species level of J. phoenicea, that's between specimens from the same population and location and among specimen from different population and location (Adams et al., 2003; Adams et al., 2006; Meloni et al., 2006).

(ISSR) Marker was successfully used to assess genetic diversity within different population of wild medicinal species (Yeh et al., 1995; Khasa and Dancik, 1996; Hsiang and Huang, 2000; Sica et al., 2005). as well it was applied on J. phoenicea species in different geographical regions. Meloni group(2006) assess the genetic variation in five mediterranean populations of J.phoenicea located in Maritime Alps, Sardinia, Asinara Island and Cyprus by three polymorphic (ISSR) primers, and found that all of the analyzed populations harbored an adequate amount of genetic variability. Also they stated that geographical isolation has represented a major barrier to gene flow in J. phoenicea.

Adams and his group (2003) also utilized (ISSR) and (RAPD) to measure concordant patterns among different genotypes by applying their study on 12 different genotype from different populations of Juniperus including J. phoenicea species, and they found that the ISSRs would be more useful for analysis of individual differences, and can be used at different organizational levels: specific, inter-specific and intraspecific level.

2.2.3. Genetic Variation Analysis

2.2.3.1. Data Gathering and Scoring

Gathering and scoring ISSR data includes running the amplifications PCRs on agarose gel, staining of the DNA using EtBr, visualization and image capture of stained DNA bands on a UV transilluminator, finally, assignment

of ISSR bands to genetic loci (Wolf, 2005). In practice, each ISSR band visualized on the gel is assigned as a locus identified by its molecular weight. This is calculated by comparing the migration of the band against DNA fragments of known molecular weight (e.g., a standardized DNA ladder) After all molecular weights have been calculated and loci assigned, the data are converted to a matrix of 1s and 0s where 1 = band present and 0 = band absent for each locus.

Because of the hypervariability in ISSR markers, it is usually possible to genotype all individuals in a study with one to three ISSR primers. With minimum number of individual = 5 per population (Wolf, 2005).

2.2.3.2. Data Analysis (Quantitative Approaches)

Studies of genetic variation (e.g., population and conservation biology) require a quantitative approach (Wolf, 2005) ISSR data can be analyzed using descriptive or comparative statistics to measure levels of diversity and can be used for describing population structure (Wolfe et al., 1998; Esselman et al., 1999; Lutz, 2001; Camacho and Liston, 2001; Culley and Wolfe, 2001; Holsinger et al., 2002; Wolfe, 2005)

The descriptive statistics often reported include the total number of genetic loci scored, the percentage of polymorphic loci, average number of bands per primer, the percentage of polymorphic bands per population, the number of species- or population-typical markers, and the number of shared and unique genotypes·

There are several method to generate genetic variation comparative statistics for dominant data, however Nei genetic distance, Φ-statistics, AMOVA, PCoA considered the main cited methods (Hsiang and Huang, 2000; Adams et al., 2003; Sica et al., 2005; Wolfe, 2005; Adams et al., 2006, Meloni et al., 2006; Gupta, 2008; Culley and Wolfe, 2011).

i. Genetic Distances

Binary genetic distances (GD) using band-matching similarity coefficients (e.g., Jaccard, or Nei and Li) might be the best technique in case when dominant marker are used to assess genetic diversity because it does not use band absences or assume Hardy-Weinberg equilibrium (Hedrick, 2000)

$$GD = n\,[1- 2n\,xy/ 2n]$$ (Nei and Li; 1979)

Here, 2nxy = the number of shared character states and n equals the total number of binary characters. When calculated across multiple loci for a given pair of samples, this is equivalent to the tally of state differences among the two DNA profiles (Huff et al., 1993; Lynch and Milligan, 1994; Peakall and Smouse, 2010).

ii. Phi Statistics (φst)

The estimation of Φ-statistics parallels the same logic as for F-statistics for codominant data, except that Φ-statistics are also estimable from binary and haploid data (Peakall et al., 1995). (Φst) widely used to characterize population genetic structure. These statistics allow the partition of genetic diversity (~ heterozygosity) within and among populations (Wright, 1946; Wright, 1951; Wright, 1965).

iii. AMOVA – Analysis of Molecular Variance

This method partitions observed variation into within and among population components using genetic distances. The among population variance component is called φ_{st}, an analog to Fst and θ (Culley and Wolfe 2001).

iv. Multivariate Analysis

Principal coordinate analysis (PCoA) is a multivariate technique that allows finding and plotting the major patterns within data with multiple loci and multiple samples (Peakall and Smouse, 2001).

PCoA allow exploring and visualizing of similarities or dissimilarities of data. It starts with a similarity matrix or dissimilarity matrix (= genetic distance matrix) and assigns for each item a location in a low-dimensional space, e.g. as a 3D graphics (Orloci, 1978). By using PCoA we can visualize individual and/or population differences, therefore inferring a useful judge about population/s structure.

GenAlEx 6.4 software (Peakall and Smouse, 2001) adequately handle dominant data such as ISSR, and used in the present study to analyze Genetic Distance, PCOA (Orloci, 1978), and AMOVA (Excoffier et al., 1992; Huff et al., 1993; Peakall et al., 1995; Michalakis and Excoffier 1996).

2.3 Essential Oils

The fragrant mixture of liquids, obtained through distillation of aromatic plant materials, is known as an essential oil (Burt, 2004). Essential oils are mixtures of fragrant substances or mixtures of fragrant and odorless substances. A fragrant substance is a chemically pure compound, which is volatile under normal conditions and which owing to its odor can be useful (Gunther, 1952).

2.3.1. Essential Oils Extraction from J. phoenicea

Essential oils of the J. phoenicea are typically isolated from two parts; leaves and berries (IUCN, A Guide to Medicinal Plants in North Africa, 2005). Most studies which focus on the essential oil of J. phoenicea have made use of hydrodistillation in Clevenger-type apparatus (Clevenger, 1928

Adams et al., 1996; Angioni et al., 2003; Cosentino et al., 2003; El-Sawi et al., 2007; Mazari et al., 2010; Adams et al., 2009; Derwich et al., 2010). As well there are other records for various herbal and medicinal species (Kulisic et al., 2004; Sokovic and Griensven, 2006; Hussain et al., 2008).

In hydrodistillation procedure, the plant material is immersed in water, which is heated to boiling point using an external heat source, the vapors are allowed to condense and the oil is then separated from the aqueous phase (Guenther, 1961). Care must be taken to ensure efficient condensation of steam, thereby preventing the loss of the more volatile oil components (Hussain, 2009).

Several studies conducted in studying different parameters that effect yield and composition of essential oil obtained by hydrodistillation; the duration of the distillation process is one important parameter that influence the yield and composition of essential oils (Koedam, 1982; Masango, 2004).The long distillation cycles should be avoided, as only a small increase in the oil yield is obtained towards the end of the cycle, also this influence more loose of polar compounds to the increased aqueous fraction (Masango, 2004).

Throughout distillation process, oxygenated compounds are released earlier from intact plant material than non-oxygenated compounds. That's because the later have lower boiling point (Erdtman and Norin, 1960; Baker et al., 2000). Polar oxygenated compounds are more water soluble than non-oxygenated compounds and thus diffuse faster and distilled first (Koedam, 1982; Baket et al., 2000). Further, Koedam (1982) found that initial distillate fractions obtained during hydrodistillation of dill seed, contained predominantly carvone (b.p. 230°C), and only small amounts of limonene (b.p. 176 °C), in spite of the latter having a lower boiling point. However, the limonene quantities gradually increased throughout the distillation, with a simultaneous decrease in carvone. Baker et al. (2000) explained that the affinity of some volatile components for lipids, prevents their release from the plant and thus it is not possible to obtain all of the oil naturally present in the plant by distillation process.

Sesquiterpenes have larger molecular size than mono and diterpenes, this is probably why it tends to distill later than oxygenated monoterpenes (Enzell and Erdtman, 1958).

2.3.2. Characterizations of Essential Oils

There is plenty of literature on the characterization of essential oils from J. phoenicea. Capillary gas chromatography (GC) with flame ionization detection (FID), are - in most cases- the method of choice for quantitative

determinations. Many researchers make use of mass spectrometers (MS), coupled with GC, to determine the identities of components (Adams et al., 1996; Angioni et al., 2003; Cosentino et al., 2003; El-Sawi et al., 2007; Mazari et al., 2010; Adams et al.,2009; Derwich et al., 2010).

2.3.3. Chemistry of Essential Oils from J. phoenicea

Gogus et al. (2005) defined essential oils as complex mixtures of organic compounds. By far the most common component class is the terpenes. Terpenes are made from combinations of several 5-carbon- base (C5) units called isoprene (Gunther, 1952).

Terpenes can form building blocks by joining together in a "head-to-tail" configuration to form monoterpene, sesquiterpenes, diterpene and larger sequences (Pinder, 1960).

Monoterpene (C10) consists of two isoprene units and is derived from the molecular formula $C_{10}H_{16}$. Sesquiterpenes (C15) consist of three isoprene units and are derived from the molecular formula $C_{15}H_{24}$. Monoterpenes and Sesquiterpenes could be only hydrocarbons or being oxygenated. Monoterpene and Sesquiterpenes also contain several functional groups like carbures (ocimene, myrcene, terpinenes, phellandrenes, pinenes, etc.), aldehydes (geranial, citronellal, etc.), ketone (menthones, pulegone, carvone, fenchone, pinocarvone, etc.), alcohols (geraniol, citronellol, nerol, menthol, carveol, etc.), esters (linalyl acetate, citronellyl acetate, isobornyl acetate, etc.), ethers (1,8-cineole, menthofurane, etc.) (Burt, 2004).

Essential oils of J. phoenicea have been reported in varying details in different countries:

In 1992 Afifi and his group identified the major constituent in Egyptian J. phoenicea of both leaf and berry oils and found that the major constituent was sabinene, and α-cedrol was the second major component in the leaf oil (31.23%) while the amount was far less in berry oil (0.47%). Also for Egyptian J. phoenicea essential oils El-Sawi and colleagues (2007) reported the yield and main components of samples collected from Sinai region. The studied sample yielded 0.36 and 1.96% for berry and leaf oil respectively, and they identified fifty eight compounds in berry oil of which monoterpenes with α-Pinene was the major constituent (38.22%), followed by sabinene (24.29%) and 66 compounds in leaf oil of which monoterpene hydrocarbon was the predominant chemical group (41.29%) with α-Pinene was the major (38.22%) followed by α -cedrol (31.23%).

In Portugal Cavaleiro et al. (2001) investigated the leaf essential oil of J. phoenicea in order to determine its major constituents. Sixty-eight compounds

were identified with α-Pinene; b-phellandrene, α-terpinyl acetate and myrcene were found to be the main constituents.

Cosentino et al. (2003) reported that α-Pinene largely predominant in the oils of the species J. phoenicea subsp. Turbinate collected from wild in Sardinia, Italy.

Dob et al. (2008) analyzed the yield and chemical composition of J. phoenicea from Algeria and reported that the sample have a yield of .8% ,and identified one hundred and three constituent of which monoterpenes hydrocarbon were the major constituent (62.2%) while oxygenated sesquiterpenes were the least (8.8%), of which α-Pinene (40%), α –phellandrene(14.7%), and Elmol (3.9%) are the main constituent .

Mazari et al. (2010) analyzed the major components of J. phoenicea essential oils from Algeria and reported a yield of 0.52% (w/w). They identified thirty six compounds and found that monoterpenes hydrocarbons are the most abundant (72.9 %) with α-pinene as the major constituent (34.5 %); β-phellandrene (22.4%) and α-Terpinyl acetate (14.7%) was the second constituent.

Derwich et al. (2010) studied the yield and chemical composition of the essential oils of J. phoenicea collected from Atlas median in the region of Boulmane (Morocco) and had yield of 1.62% (w/w), also they identified twenty three compounds of the leaf oil. α-pinene was the major constituent (49.15%). followed by α-phyllandrene (7.39%) and myrecene (5.24%).

In a study conducted in Greece, Spain, Morocco, Madeira and the Canary Island, Adams and his group (1996), (2001) and (2009) showed that monoterpenes majored by α-Pinene represented the major constituent in all studied populations of J. phoenicea.

Medini et al. (2011) examined the chemical composition of the essential oil of Juniperus phoenicea L. both of ripe and unripe berries from Tunisia. the study revealed the detection of 42 components represented approximately 96.50–99.57% of the oils of which the major components the were α-pinene (58.61–77.39%), camphene (0.67–9.31%), δ-3-carene (0–10.01%) and trans-verbenol (0–5.24%). Also they confirmed that quantitative variation regard oil composition according to the ripening stage of the berry was present.

2.4 Factors Affecting Essential Oil Accumulation

Thier are several factors that influence the composition and yield of the essential oil. These factors may include seasonal and maturity variation, geographical origin, genetic variation, growth stages, part of plant utilized and postharvest drying and storage (Zobayed 2005; Adams and Nguyen, 2006; Hussain et al., 2008; Figueiredo et al., 2008). Moreover it is difficult to

segregate these factors from each other, since many are interdependent and influence one another (Terblanche, 2000).

This study brings into focus the influence of part of tree utilized for extraction, genetics and geographical variation into essential oil accumulation in J. phoenicea essential oil.

2.4.1. Part of plant used

Essential oils of J. phoenicea are mainly extracted from its leaf or berry like fruit. Physiological factor that appears when different part of plant is used to obtain the oil were observed and recorded in many studies:

In 1992 Afifi and his group identified the major constituent in Egyptian J. phoenicea of both leaf and berry oils and found that the major constituent was sabinene and α-cedrol was the second major component in the leaf oil (31.23%) while the amount was far less in berry oil (0.47%).

Angioni et al. (2003) investigated the composition of the essential oil from J. phoenicea berries and leaves from Sardinia (Italy) The yields ranged between 2.54% ± 0.21 (v\w dried weight) and 0.04% ± 0.00 and they successfully identified 36 components among of which α-pinene, β-pinene, δ-3-carene, sabinene, myrcene, β-phellandrene, limonene, and D-germacrene were The major constituent. However they reported the presence of qualitative and quantitative differences between different parts of the plant regard the yield and the composition of the oil. Further, they recorded a variation in the essential oil composition of the berry oil as obtained from ripe and unripe berries.

El-Sawi et al. (2007) investigated the oil composition and yield from leaf and berry of J. phoenicea and found that quantitative and qualitative differences are present as fifty eight compounds identified in berry oil and α-Pinene was the major 38.22%, followed by sabinene (24.29%) while leaf oil was composed of about 66 compounds. α-Pinene was the major (38.22%) followed by α -cedrol (31.23%).

Ennajar et al. (2009) analyzed essential oils of Tunisian Juniperus phoenicea both from leaves and berries. Their study led to the identification of 30 compounds, representing more than 98% of the total composition. Quantitative variation were observed among the studied samples as α-pinene (55.7% and 80.7%), δ-3-carene (10.7% and 4.5%), and γ-cadinene (2.9% and 5.1%) were the main components, in leaves and berries essential oil respectively.

2.4.2. Genetic factor

Genotype is typically defined as "the genetic make-up of an organism, as characterized by its physical appearance or phenotype", while chemotype is generally defined as "a group of organisms that produce the same chemical profile for a particular class of secondary metabolites". Genetic makeup of the plant is one of the most intrinsic contributors to their essential oil composition (Graven et al., 1990).

Variations in chemical profiles were observed from oils produced by specimens from the same population of J. phoenicea (Cavaleiro et al., 1996). Also a significance variability of the leaf essential oil observed among different population and location, demonstrating the presence of different chemotypes within this species.

In (1996) Adames anad coworkers analyzed and compared the entire leaf oil composition of J. phoenicea var. phoenicea and J. phoenicea var. turbinate both from Spain based on this they differentiated two group of the oil in which J. phoenicea var. turbinate characterized by high α-pinene, β-phellandrene, α-terpinyl acetate with no limonene and low myrecen, and J. phoenicea var. phoenicea which characterized by high α-pinene and moderate amount of β-phellandrene, limonene and α-terpinyl acetate was missing .

Rezzi et al. (2001) reported on infraspecific variation in the leaf essential oils of J. phoenicea var. turbinata from Corsica. They found two chemical types: high α-pinene, low β-phellandrene, low α-terpinyl acetate (cluster I, 35 indvs.); and low α-pinene, high β-phellandrene, high α-terpinyl acetate (Cluster II, 15 indvs.) without any morphological differences been observed .

Cavaleiro et al. (2001) investigated the essential oil composition of 68 individual plants of J.phoenicea var. turbinata from Portugal and stated that variability of the leaf essential oil contribute for the taxonomy and characterization of infraspecific variability of J. phoenicea. The results of the analysis showed that this taxon exhibits chemical polymorphism as three groups of essential oils were present depending on the content of α-pinene, β-phellandrene and α-terpinyl acetate.

2.4.3. Geographical Influnce

Variation into essential oil content among different geographical regions could be associated to variation in soil textures and composition, possible adaption response (Hussain et al., 2008), Altitude (Vokou et al., 1993), Climatic factors such as heat and drought (Viljoen, 2006; Milos et al., 2001). Such geographical variation among different populations of the same plant species, resulting in different chemical profile, this is may be without any

morphological differences being observed in the studied species (Hussain et al., 2008).

Thier are many reports in the literature showing the variation in the yield and chemical composition of the essential oil obtained from J. phoenicea with respect to geographical regions: Adams and his colleagues (1996) reported the presence of some qualitative and quantitative variation in yield and chemical composition of essential oils from different countries: Greece, Spain and Portugal, despite that each of the oil are dominated by α-pinene, Greece sample exhibit high β-phellandrene, limonene and α-terpinyl acetate content while limonene and α-terpinyl acetate was missing in spain oil samples. In addition some unknown compounds were present in Portugal and Spain sample that's missing in Greek sample.

Adams et al. (2009) evaluated the variation and systematic of leaves volatile oils of J. phoenicea population obtained from Madeira and the Canary Island, also compare it with populations from Spain and Morocco and found that all of the oils of J. phoenicea from the Canary Islands and Madeira were very similar yet there is some differentiation in this oils from from populations in Spain and Morocco as was the oil from Morocco contain (65.4%) α-pinene, and is higher than in J. p. var. phoenicea, Spain which contain (41.2%) α-pinene or var. turbinata, Spain, with (25.8%) α-pinene. On the other hand the Madeira and Canary Island oils had (57.3 - 76%) amounts of α-pinene, also the oil from Morocco was the only oil with camphor (1.3%). on addition, they reported that the oil of J. p. var. phoenicea, Spain, contained a large concentration of manoyl oxide (22.0%). The oil of J. p. var. turbinata, Spain, contained large amounts of β-phellandrene (31.5%) and α-terpinyl acetate (13.1%) along with the smallest amount of α-pinene (25.8%).

Derwich et al. (2010) studied the yield and total oil composition of the essential oils of J. phoenicea collected from Atlas median in the region of Boulmane (Morocco) and compared that with other records about J. phoenicea leaf oil yields obtained from other countries; Portugal, Spain, Greece (Adams et al., 1996) and Egypt (Elsawi et al., 2007). They stated that quantitative variation is presents in both the yield and total oil composition (%) as from Morocco was 1.62 and 81.87 respectively. in comparison it was 1.96and 99.16 from Egypt, 0.58 and 88 from Greece, 0.66 and 99 from Spain, 0.41and 98.3 from Portugal for yield and total oil composition (%) respectively.

2.4.4. Soil Type and Composition

The soil type and its composition considered one of the main external factors, which can change the concentration of secondary metabolites and its active constituents such as essential oil in medicinal plants and maintaining its

biodiversity (Figueiredo et al., 2008; Aziz et al., 2008). Nitrogen (N), phosphorus (P) and potassium (k) are of the main nutrients required for plant growth.

Nitrogen availability in soil thought to affect many characteristics of the medicinal plants such as the concentrations of secondary active compounds (Letchamo et al., 1995). The plant growing in nitrogen-poor condition is thought to contain more secondary metabolites compounds than plants growing in a nitrogen-rich environment this is according to the carbon/nutrient balance hypothesis (Bryant et al., 2008). Carbon/nutrient balance hypothesis includes that when nitrogen availability in the soil is low, the low resource availability limits the growth of the plants more than the photosynthesis, and plant allocates the extra carbon that cannot be used for growth to the production of carbon based secondary metabolites. This hypothesis is supported with multiple studies on different medicinal species: Nitrogen fertilization has been reported to reduce volatile oil content in Juniperus horizontalis (Fretz, 1976), Wen-Hua et al. (2009) found that the contents of secondary metabolites as flavonoids and caffeates in medicinal plants were negatively correlated with soil nitrogen availability. Similar result were recorded by Ibrahim et al.(2011) as found that low nitrogen levels in the soil was correlated with Enhanced Production of total flavonoids (TF), and total phenolics (TP) in Labisia Pumila Blume species.

Phosphorus is a central and pivotal metabolic and regulatory nutrient element in different physiological and biochemical processes in plant, including photosynthesis, energy conservation, inter- and intracellular co-ordination of carbohydrate metabolism (Abel, 2002) and in energy transfer (Harley, 1971). Saharkhiz and Omidbaigi, (2008) recorded that increasing phosphorus level in the soil correlated with significant enhancement of growth parameters and essential oil concentration and yield in feverfew (Tanacetum parthenium L.) Which is an important medicinal plant in Iran, Similar results have been noted by another investigator for different medicinal species such as sweet basil, black cumin (Nigella sativa), coriander (Corianderum sativum), and geranium (Pelargonium species) (Das et al., 1991; Ichimura et al., 1995; ghreja and Chundawat, 1992)

Potassium is a key essential plant nutrient although it is not a constituent of any plant part. It acts as catalyst for many of the enzymatic processes which are necessary for plant growth. It also regulates the opening and closing of stomata which affect carbon dioxide uptake for photosynthesis (Somida, 2002). Different worker reported that Potassium contribute in enhancement of essential oil production in different medicinal species e.g.; Oregano

(Origanum vulgare L., and Caraway (Carum carvi L.) (Said-Al Ahl et al., 2009; Ezz El-Din et al., 2010).

Many researchers studied the effect of soil salinity on essential oil and chemical composition for several medicinal species i.e. Abou El- Fadl et al.(1990) indicated that by increasing the soil salinity essential oil and its components were increased in peppermint plant. Khalid (2001) reported that, plant growth of (Nigella sativa) was significantly decreased by increasing the levels of soil salinity but oil and its components were increased. Hendawy and Khalid, (2005) demonstrated that increasing salinity level in soil led to decreased plant growth and nutrient content but essential oil, total carbohydrates and proline content were increased in (Salvia Officinalis L.). Baghalian et al. (2008) showed that chamomile is able to maintain all its Secondary metabolites and medical properties, under saline condition and could be cultivated economically in such conditions.

2.5 Antimicrobial activities

2.5.1. Antimicrobial agents

In general Antimicrobial agents could be defining as substances that acts to slow the growth of harmful microorganisms or act to destroy them (Brock et al., 2012).

Antimicrobial agents differentiated into two main types: 1) Antibiotics, that are natural substances produced by certain groups of microorganisms and 2) chemotherapeutic agents, which are chemically synthesized (Davidson and Harrison, 2002). It is desirable to use an antimicrobial agent that can inhibit a wide range of infection-causing microorganisms, but this is hard to achieve. Also, a good understanding of the chemicals' mode of action is useful in selecting a certain antimicrobial agent.

2.5.2. Essential oils as Antimicrobial agents

Various side effects are associated with the use of drug antibiotics, this is mainly because many pathogens have developed a resistance against them (Nester et al., 2007). Many multidrug resistant strains of bacteria and other with reduced susceptibility to antibiotics have appeared. As a result the specter of untreatable bacterial infections has been raised (Sieradski et al., 1999). This is due often due to the wide spreads of antibiotics and the uncontrolled or indiscriminate use of them (Brock et al., 2012). In addition to that, antibiotics are sometimes associated with adverse effects on host, such as, hypersensitivity, depletion of gut and mucosal floral microorganisms, immunosuppression and allergic reaction (Idose et al., 1968). This problem calls for the search for alternative infection-fighting strategies.

In the past few decades interest about aromatic plant and their essential oil extract have been raised as antimicrobial agents, although it was long been used for a wide variety of medicinal and domestic purposes (Knobloch et al., 1989). On addition, Investigation about Plants active constituents has been the subject of many research and oils with very potent antibacterial and antifungal activity could be promising agents for the future more extensive research and in vivo examination (Pepeljnjak et al., 2005).

Essential oils from Juniperus phoenicea were tested for its antibacterial activities against a wide spectrum of Gram-positive and Gram-negative bacteria (Angioni et al., 2003; Cosentino et al., 2003; El-Sawi et al., 2007; Derwich et al., 2010; Mazari et al., 2010) as well as its antifungal and antioxidant activity (Ennajar et al., 2009). For example, it was tested against Staphylococcus aureus, Enterococcus feacalis, Aspergillus flavus, Fusarium oxysporum and Rhizopus stolonifer.

Angioni et al. (2003) tested the antimicrobial activity of ripe and unripe berries and leaves of three Juniperus sp, including J. p. var. phoenicea from Italy, against C. albicans, S. aureus, E. coli, and P. aeruginosa. The obtained data led to a non significant inhibitory effect, although all the essential oils from leaves exhibited rather good or weak activity against C. albicans and S. aureus.

Cosentino et al. (2003) investigated the antimicrobial properties for berry essential oils obtained from J. phoenicea subsp. Turbinate from wild in Sardinia (Italy) against food spoilage and pathogenic microorganisms. Thier results showed that the oils extracted from J. phoenicea subsp. turbinata specimens were active against fungi, particularly against a strain of Aspergillus flavus (an aflatoxin B1 producer). The single compounds tested, δ-3-carene was found to possess the broadest spectrum of activity and appeared to contribute significantly to the antifungal activity observed for J. turbinata oils.

El-Sawi et al. (2007) investigated the anti microbial activity of J.Phoenicea oil obtained from leaf and berry against bacteria (E. coli, S. aurous B. subtilis and B. cereus) and antifungal activity against (Fusarium oxysporium, Aspergillus niger, Aspergillus flavus, Maccrophomina phasioli, Botryties allii Trichoderma, veridus) and Yeast (Candida pseudotropicalis, Candida albicans, Candida carlspergensis, Saccharomyces cerevisiae, Saccharomyces chevallii Rhodotorula minuta). They stated that the leaf oil has higher activity than berry oil against E. coli, B. subtilis and B. Cereus. The highest activity was against B. subtili and yeasts as it recorded the lowest MIC value but it was inactive against S. aureus while berry oil was inactive against two bacterial strains; S. aureus and B. cereus.

Mazari et al. (2010) investigated the antimicrobial activity of Algerian J. phoenicea for leaf essential oil and found that it possessed antimicrobial activity against five bacteria (S. aureus, E. feacalis, B.cereus, E. coli, and P. aeruginosa), and three fungi. (Aspergillus flavus, Fusarium oxysporum and Rhizopus stolonifer). Their Results showed that the oils exhibited moderate antibacterial and antifungal activities, and E. feacalis was the most sensitive microorganism with the highest inhibition zone (15.6 mm) and lowest MIC value (7µl/ml).

The antibacterial activity of Moroccan J. Phoenicea leaf essential oil was described by Derwich et al. (2010). They stated that leaves oil was very effective against seven bacterial strains: E. coli, S. aureus, S.intermedius, K. pneumonia, P. aeruginosa, B. subtilis and S. mutans. (MIC) ranging from 0.02 to 0.40 mg per ml. also they found that E. coli was the most sensitive strain tested with the strongest inhibition zone (34 mm).

a Correlation between chemical composition and antimicrobial activities of essential oils extracted from from J. phoenicea also have been mentioned on the previous recordes. Most of the activity appears to be explainable by terpenes composition. Glisic et al. (2007) evaluate the antimicrobial activity of the essential oil and different fractions of Juniperus communis L. and found that the native oil showed weak antimicrobial activity, while the fractions with a high content of α- pinene, and mixture of pinene and sabinene showed the highest antimicrobial activity, especially against fungi.

The major terpenes in J. phoenicea oil such as, α-pinene, α-Terpinyl acetate and cedrol has been known to exhibit antimicrobial activity against different bacterial strains among of which E. coli, S. aureus, B. cereus, and B.subtilis (Cosentino et al., 1999; Dorman et al., 2000; Glisic et al., 2007; Bourkhiss et al., 2007; Derwich et al., 2010). Monoterpenes hydrocarbons, have shown strong to moderate antibacterial activity against Gram positive bacteria in many reports (Delaquis et al., 2001; Kim et al., 2003; Oyedeji and Afolayan, 2005).

2.5.3. In Vitro tests of antimicrobial activity

Numbers of methods used for evaluation of antibacterial activity of essential oils and extract obtained from J.Phoenicea have been reported in literature (Angioni et al. 2003; Cosentino et al. 2003; Hayouni et al. 2007; El-Sawi et al. 2007; Mazari et al. 2010; Derwich et al. 2010; Fouad et al. 2011). Different protocol like disc diffusion assay, hole plate diffusion assay, Microdilution assay, measurement of minimum inhibitory concentration are often used .

In disc diffusion assay paper disk soaked with known concentration of essential oil laid on top of an inoculated agar plate. This is generally used as a preliminary check for antibacterial activity prior to more detailed studies. A number of factors such as the amount of essential oil placed on the paper discs and the thickness of the agar layer vary considerably between studies (El-Sawi et al. 2007; Mazari et al. 2010; Derwich et al., 2010)

On the other hand many reports make selection of hole plate diffusion assay for anti microbial activity evaluation purposes (Daud et al., 2005; Glisic et al., 2007; Rezvani et al., 2009 and Ichrak et al., 2011). In this method holes are formed by cutting into agar by appropriate cylinder and the essential oils are loaded to that holes. Inhibition is determined by measuring clearance zone diameters formed around the hole. For example it is possible from knowledge of the zone diameters produced by two different substances, and of the time of diffusion involved, to estimate its ability to inhibit the growth of certain microorganism (Humphrey and Lighfbown, 1952). The homogeneity of the tested organism's population with a sharply defined concentration is critical factors concerned in determining the zone edge, since it becomes clear that the ideal of a perfectly sharp zone edge produce more accurate determination of the inhibition (Humphrey and Lighfbown, 1952).

One of the most cited and important method in the antimicrobial performance of essential oils is the measurement of minimum inhibitory concentration (MIC) which define as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of microorganisms (EUCAST, 2000). In most cases (MIC) gives us accurate, exact and reproducible results. In some cases, the minimum bactericidal concentration (MBC) or the bacteriostatic concentration further applied, both terms agreeing closely with the MIC. The strength of the antimicrobial activity can be determined by dilution of essential oils in agar or broth (Ericsson and Sherris, 1971; Pintore et al., 2002). However, Agar dilution considered the golden standard method and reference in determining (MIC) (EUCAST, 2000; Hendriksen, 2003).

The review of past studies as has been discussed in this chapter revealed that essential oils obtained from J.phoenicea have gained much appreciation among microbiologist and food scientist and researchers because of their multifold biological activities. Although J.phoenicea have been investigated for its essential oil and its potential biological activities and genetic variation, however, to the best of our knowledge there are no earlier reports yet available regarding the detailed chemical characterization and evaluation of biodiversity from J.phoenicea, native to Jordan.

CHAPTER THREE
Material &methods

The research work presented in this thesis was conducted in the laboratories of the Department of biology at Al-Hussein Bin Talal university, Maan, Jordan; National Center of Agricultural Research (NCAR), AL-Balqa, Jordan; Princess Feisal center of dead sea research, University of Mu'tah, AL-Karak, Jordan; Department of pharmaceutical science, University of Jordan, Amman, Jordan.

3.1. Materials

Primer purchased from IDT Integrated DNA technology, (I-MAX) PCR ready mix solution from (iNtRON, South Korea).

.1. authentic standards ; α-thujene, α-pinene, β-pinene, p-cymene, limonene, β-phellandrene, γ-terpinene, terpinolene, linalool, borneol, terpinen-4-ol, α-terpineol, nerol, geranial, thymol, carvacrol, eugenol, β-caryophyllene, aromadendrene, α-humulene, caryophyllene oxide, δ-cadinene, and γ-cadinene (purchased from Sigma-Aldrich)

2. Culture media; nutrient broth, and nutrient agar, (Scharlaue, Barcelona-Spain).

3. Standard antibiotic disks; Chloramphenicol (30 µg\disk), Tetracyclin (30 µg\disk), Ampicillin (10 µg\disk), Tobromycin (10 µg\disk) and Gentamycin (10 µg\disk) purchased from (Mast Diagnostic, UK)

Reagents and chemicals (analytical grade): Dimethylsulfoxide (DMSO), Ethanol (GC-grade), n-hexane (Scharlaue, Barcelona-Spain), MgCl2, Tris – HCl, EDTA, NaCl2, isopropanol all are purchased from Merck (Darmstadt, Germany) Except that for Agarose (molecular grade) purchased from Sigma, united state.

3.1.1 Instruments

The instruments used for different analyses during the study along with their company identification are listed in Table 3.1.

Table 3.1
Instruments used with their model and company

Name of instrument	Manufacturing Company & Modle
Clevenger	-
GC/MS	(Varian chrompack CP-3800)
GC	(DB-5)
Spectrophotometer (Smart spec.)	Bio Rad, USA
Spectrophotometer	(bio wave WPA- 52100)
UV Laminar air flow (Logic)	LABCONCO, USA
Incubator (ID-90)	Rypa, Spin
Shaker incubator	Shel Lab, Germany
Ultra low Freezer (Lab Tech)	DIHAN, Japan
Electric Balance (AAA-250L)	Adams, UK
Water Bath with shaker (W116-230)	Lab net, USA
Magnet Stirrer , Vortex , Autoclave	-
Centrifuge	-
Thermal cycler	(MJ Research -PTC-2000)
Gel electrophoreses apparatuses	Bio Rad, USA
UV transilluminator	-

3.1.2. Plant materials

The plants used in this study were sampled from five natural Populations of Juniperus phoenicea L. located in S.Jordan. The Geographical map (Figure 3.1) shows the location of the five populations of Juniperus phoenicea studied and their characteristics are reported in Table 3.2.

All the samples are collected during May, 2011 and further identified by Dr. Maha Elsyof at gene bank unit, Jordanian national center of agricultural research (NCARE).

Collection was made according to the purpose of the study:

i. Both twigs and green unripe berries were collected from each region in order to study the chemo diversity and antimicrobial activity. The collected material has also been deposited in Herbarium Department of (NCARE).

ii. Newly growing leaves were used to study the genetic diversity among the studied populations. These bud materials were frozen in liquid nitrogen until DNA extraction.

Table 3.2
Geography of five natural populations of Juniperus phoenicea used in the study

Population	Latitude (N)	longitude (E)	Altitude (m)	No. of Individual
1 Petra (P)	30. 4274	35. 4479	990	17
2 Al-taybeh (T)	30.25844	35. 45696	1100	7
3 Al –Shoubak (S)	30. 47152	35. 49953	1370	7
4 Dana (D)	30.64981	35. 60841	1206	17
5 Wadi Rum (W)	29. 57444	35.41722	1734	15

The climate in the studied area generally is semi arid to arid and annual rainfall ranging from 200 to 350mm with large variability between and within the regions in the table above.

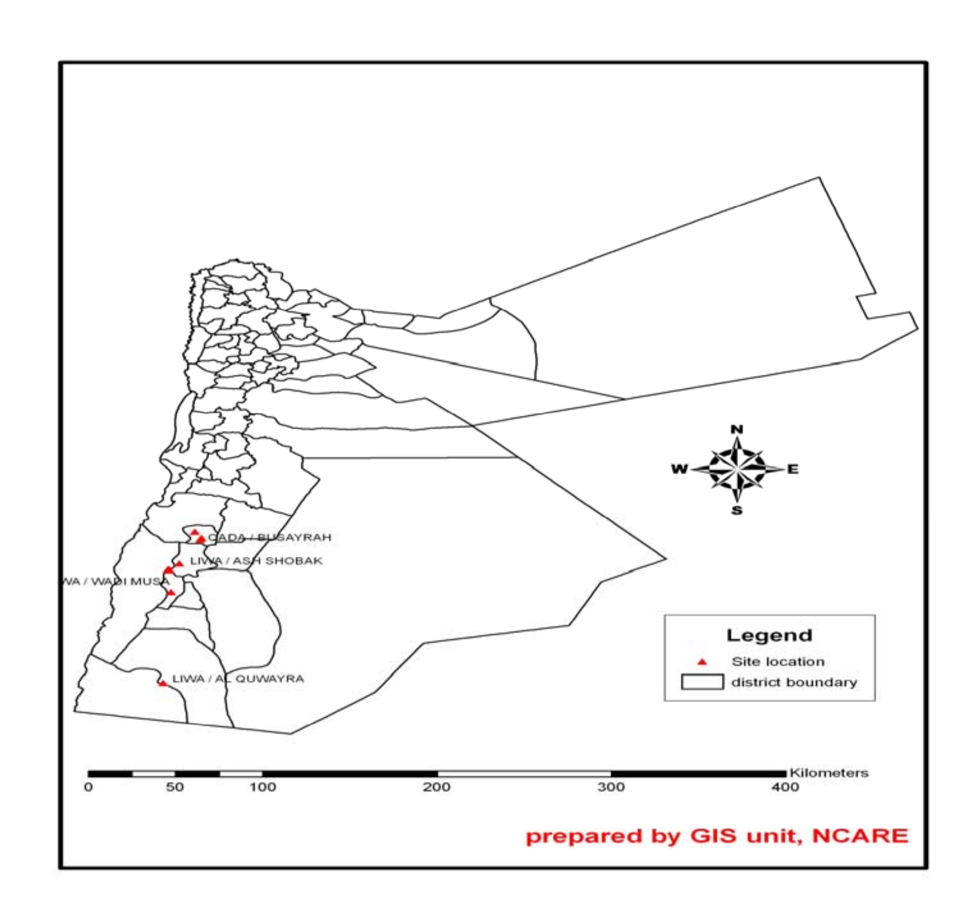


Figure 3.1. Geographical map show the location of the five populations of J. phoenicea L. from S. Jordan

3.1.3. Sequences of the ISSR primers utilized in the genetic variation study

Table 3.3.
Sequences of the 16 ISSR primers used in the Preliminary screening on the five populations of J.Phoenicea (The three primers used for genotyping are indicated in bold)

ISSR	primer sequence 5′ →3
ISSR(AG)8–1	agagagagagagagagTC
ISSR(AG)8–2	agagagagagagagagGT
ISSR(AG)8–3	agagagagagagagagGC
ISSR(AG)8–4	agagagagagagagagCA
ISSR(AC)8–1	**acacacacacacacacAG**
ISSR(AC)8–2	acacacacacacacacCG
ISSR(AC)8–3	acacacacacacacacGA
ISSR(AC)8–4	**acacacacacacacacTG**
ISSR(AT)8–1	atatatatatatatatTT
ISSR(AT)8–2	**atatatatatatatatGC**
ISSR(AT)8–3	atatatatatatatatCT
ISSR(AT)8–4	atatatatatatatatAG
ISSR(CT)8–1	ctctctctctctctctAC
ISSR(CT)8–2	ctctctctctctctctGC
ISSR(CT)8–3	ctctctctctctctctAG
ISSR(CT)8–4	ctctctctctctctctGT

3.1.4. Strains of microorganisms utilized to access the antibacterial activity of essential oils

Antimicrobial activity tests were carried out against two Gram-positive: Staphylococcus aureus (SLV-350), Bacillus cereus (ATCC 10875) and two Gram-negative: Escherichia coli (ATCC 0175) and Pseudomonas aeruginosa (ATCC 27853).

The bacterial strains were supplied by Aqaba international laboratories (Ben hayyan), and were maintained refrigerated in Mueller Hinton agar plate until used.

For inoculums preparations, bacteria were sub-cultured in nutrient broth at 37˚C Until reach log phase and then adjusted to a suspension of 1×10 8 CFU/ml (absorbance at 600nm=.200)

The optical density (OD) at 600 nm of each culture was measured with a Spectrophotometer.

3.2. Experimental Protocols

3.2.1. Genetic variation among and within five population of J. Phoenicea

Genetic variation of J.Phoenicea from the southern Jordanian forest were assessed as a preliminary study both at inter and intra population level using ISSR marker.

3.2.2. Effect of part used for extraction in the quantity and quality of essential oil and antibacterial property

Effects on the plant part that used on chemical composition, and antibacterial activities of essential oils from selected population of J.phoenicea were investigated. The specimens were washed with tap water and then dried at 30˚ C in a hot air-oven.

3.2.3. Geographical variation in the quantity and quality of essential oils and its antibacterial property

The chemical composition, and antibacterial activities of the essential oils isolated from selected specimen from different geographical regions in S.Jordan was investigated. The specimens, collected in clean polythene bags were brought to lab washed with tap water and then dried at 30 ˚C in a hot air oven.

3.2.4. Comparison between J. phoenicea essential oil activities with some commercial antibiotics

Oil with the best inhibition zones in this study were compared with five types of commercial antibiotics (section 3.1.1) to further investigate if J. phoenicea essential oil can be employed in place of some antibiotics against some food born bacteria (3.1.5).

3.3. Isolation of Essential Oils

The essential oils of leaves and berries were individually extracted. Representative sample from both were used from three regions of Juniperus phoenicea namely; Wadi Rum (W), Petra (P) and Dana (D).

The essential oils were extracted by hydro-distillation using an apparatus of Clevenger type. The extraction took 3 h for mixing 200 g and 250 g of fresh needles and crashed berries respectively in 1000 mL of distilled water. Pure oil was stored at 4°C in obscurity till the beginning of analysis. The essential oils yield was demonstrated by the oil quality (in mL) obtained for 100 g of plant sample.

3.4. Gas chromatography-Mass spectrometry (GC-MS) analysis

Gas chromatography-Mass spectrometry (GC-MS) analysis was done by the Technician Staff of GC/MS lab. In Jordan University, Faculty of Pharmacy.

3.4.1. Sample preparation for (GC-MS) analysis

5 μL from the invistgated oil was diluted up to 1 ml using GC-grade n-hexane, and then 1 μL samples of the diluted oil were injected into the GC-MS systems for analysis.

3.4.2. GC-MS systems and analysis

The GC-MS systems is Varian Chrompack CP-3800 GC/MS/MS-200 equipped with split-splitless injector and DB-5 GC column (5% diphenyl 95% dimethyl polysiloxane, 30 m × 0.25 mm ID, 0.25 μm film thickness). The injector temperature was set at 250°C with a split ratio of 1:10. Detector and transfer-line temperatures were 160°C and 230°C respectively. A linear temperature program was used to separate the different oil components. Temperature programming was applied based on the mentioned temperature. The mass detector was set to scan ions between 40-400 m/z using full scan mode and electron impact (EI, 70 eV). A hydrocarbon mixture of n-alkanes (C8-C20) was analyzed separately by GC-MS using the same column (DB-5) and under the same chromatographic conditions. For each component (peak) separated by GC-MS, the linear retention index (arithmetic) was calculated as according to Van den Dool and Kratz (1963).

3.4.3. Identification of compounds

Identification of oil components was performed by matching their spectra with the data bank mass spectra (WILEY, NIST and ADAMS-2007 libraries) and also by comparing their linear retention indices with reported values in the literature, mainly the Adam's library (2007). Identification of certain compounds (e.g. α-thujene, α-pinene, β-pinene, p-cymene, limonene, β-phellandrene, γ-terpinene, terpinolene, linalool, borneol, terpinen-4-ol, α-terpineol, nerol, geranial, thymol, carvacrol, eugenol, β-caryophyllene, aromadendrene, α-humulene, caryophyllene oxide, δ-cadinene, and γ-cadinene) was further confirmed by co-chromatography of their authentic standards (purchased from Sigma-Aldrich) under the same chromatographic conditions mentioned at Section 3.4.2.

3.5. Evaluation of the Antibacterial activity of the essential oil

3.5.1. hole-plate diffusion assay

The essential oils of leaves and berries were individually tested. Representative sample from both were used from three locations of J.phoenicea namely; Wadi Rum (W), Petra (P), Dana (D). The Antimicrobial screening protocol was carried out by the "hole-plate diffusion method". The tested bacterial suspension was homogeneously spread onto a surface of 9.0cm Petri dishes containing 20 ml of the Nutrient agar (NA) agar medium. The medium was prepared by dissolving 28g (NA) in 1L distilled water and were sterilized by autoclave for 20 min at 120 ° C. two Holes were aseptically bored into the each agar plate using 6mm cork borer. Subsequently 50µl aliquots of the oil were placed into each hole with a sterile pipette. The plate was kept for 1 h at room temperature to facilitate the diffusion of the oil into the agar. Subsequently, the plate was incubated at 37 º ± 1°C for 18 h.

Standard antibiotic disk: Chloramphenicol (30 µg\disk), Tetracyclin (30 µg\disk) ampicillin (10µg\disk), Tobromycin (10µg\disk) and Gentamycin (10 µg\disk), were used as positive control, whereas dimethyle sulfoxide (DMSO) was used as negative control. The microorganism control consisted of a petri dish with solvent (DMSO) or standard antibiotic disk instead of oil sample.

Results were recorded as the mean of replicated two trail experiments. Bacterial Growth Inhibition was determined as the diameter (in millimeters) of the inhibition zones around the holes measured by transparent ruler. The inhibition Diameter was the average of four measurements per hole.

3.5.2. Determination of Minimum Inhibitory Concentration (MIC)

For the determination of MIC, which defined as the lowest concentration at which the microorganism does not demonstrate visible Growth; agar Dilution method was used as described by (EUCAST, 2000), and it was determined for only those sample that showed significant antimicrobial activity against the test microorganisms (section 3.5.1). Media were prepared by dissolving 28g (NA) in 1L distilled water and were sterilized in an autoclave for 20 min at 120 ° C. then the sterilized agar allowed to cool to 50 ºC in a water-bath.

The essential oil extract (Section 3.3) was diluted by adding 20, 40, 60, 80, 100, 125, 150, 200, 250, 300, and 400 µl of the extract to 980, 960, 940, 920, 900, 875, 850, 800, 750, 700 and 600 µl of 0.2% (w/v) agar solution, respectively. Emulsification was carried out by aseptically adding 1 ml of the diluted essential oil extract to 19 ml of sterile culture medium and vortex to disperse the oil (ABI-Ayad et al., 2011). The final concentration of each essential oil extract obtained was 1, 2, 3, 4, 5, 6.25, 7.5, 10, 12.5, 15 and 20

µl/ml respectively. The agar was then poured into sterile petri dishes. The control agar plate consisting of 19 ml of culture medium and 1 ml of 0.2% (w/v) agar solution.

Preparation of the inoculums

Preparation of the inoculums was also performed as described by (EUCAST, 2000). The assay Plates were inoculated using a sterile micropipette to transfer about 1 µL Inoculate per spot of each tested micro organisms. The density of the inoculums was previously standardized photometrically to give (10^8) CFU/mL, and Four to five spot were made per each plate. The tests were carried out in replicate and MIC was exhibited after 18 hrs incubation at 37±1° C.

3.6. Statistical analysis for antimicrobial activity

Two samples of each isolated oils were assayed. Each sample was analyzed individually in replicate for its antimicrobial activity and data is reported as mean ± standard error of mean. Data were analyzed by analysis of variance (ANOVA) at 5% significance level.

3.7. DNA Extraction

Total genomic DNA was extracted from 100 mg tissue of each plant Samples using CTAB method according to the protocol described by (Pirttila et al., 2001):

(i) The tested plant bud material was grinded in a mortar and pestle with liquid nitrogen.

(ii) One hundred milligrams of the resulted fine powder tissue was placed in 1.5 ml microfuge tubes together with 700 µl of the prewaremed CTAB buffer.

(DNA extraction buffer (CTAB) was prepared according to the following recipe: 2% cetyltrimethyl-ammoniubromide (CTAB) (w/v), 100 mM Tris – HCl (pH 8), 20 mM EDTA (pH 8), 1.4 M NaCl2; mixed then autoclaved).

(iii) The tubes were vortexes thoroughly and holed at 65º C water bath for 10 min with continuous mixing.

(iv) Tubes were cooled to room temperature and 700 µl of chloroform – Isoamyle alchole (IAA) (24:1) were added to each tube and vortexed thoroughly for a few Seconds.

(v) The tube were then Centrifuged at 13.000 rpm for 5 min.

(vi) The upper phase of each sample was transferred into a new sterile tube and extracted with 700 µl chloroform – IAA and centrifuged.

(vii) The upper phase of each sample was transferred into a new sterile tube and equal volume (about 600 µl) cold isopropanol were added.

(viii) The tubes then were inverted carefully several times to mix the two layers, and placed at -20º C for 30 min.

(ix) Then the tubes were centrifuged at 13.000 rpm for 10 min at 4ºC.

(x) Supernatant were discarded and pellets were then dissolved in 300 µl distilled autoclaved water.

(xi) About two volume (600 µl) cold absolute ethanol was added to each tube.

(xii) Then the tubes were placed at -20º C for 1 h, and recentrifuged at 13.000 rpm for 10 min at 4ºC.

(xiii) The Pellets were collected and washed with 300 µl of 70% cold ethanol then recentrifuged at 13.000 rpm for 5 min and left for air drying.

Finally**,** DNA pellets were Dissolved in 50 µl sterile distilled water and stored at -20 until PCR reaction.

The quality and the concentration of the DNA were checked and standardized by spectrophotometer.

3.7.1. ISSR amplification

An initial set of 18-mer 16 primers **(**Table 3.3) was screened to select those generating good amplification patterns. Primers were chosen as described by Meloni and colleagues (2005). In which they are some of the frequent dinucleotide microsatellites in plants flanked by a dinucleotide sequence 3 anchored.

PCR amplifications were performed in a final volume of (20 µL) containing (30) ng of genomic DNA, (1–2.5) µM of MgCl2, about 2 µl of the primer and (15 µL) PCR ready mix solution.

3.7.2. PCR Condition

PCR reaction carried out on using the following profile: 94 ºC for 4 min followed by 45 cycles of 94 ºC for 30 s, 52 ºC for 45 s, 72º C for 2 min and ended with 72º C for 7 min.

All amplification reactions were repeated at least three times and only 3 primers exhibited specific PCR products unequivocally scorable and reproducible in Successive amplifications were used for the final study (Table 3.4).

Table 3.4
Amplification parameters and number of reproducible Bands for each of the three ISSR primers used in this study

ISSR	annealing temperature (C)	No. of loci detected
ISSR(AC)8–1	52	10
ISSR(AC)8–4	52	13
ISSR(AT)8–2	52	12

3.7.3. Data collection

After amplification, 15 microliters of the 20 microliter products were run out on a 1.7 % agarose "mini-gel" electrophoresis apparatuses and stained with ethidium bromide included in the agarose at the concentration of 15 microliters per 100 ml agarose. Agarose gel run at 3Vcm_1 in Tris-EDTA-Acetic Acid (TEA) 1X buffer just until the tracking dye has traveled about 1/3 of the gel length. Then Gels were Visualized on a UV lamp and photographed. Molecular weights were estimated by comparison with a 100-bp DNA ladder. PCR products were scored for band presence or absence.

3.7.4. Data analysis

ISSR bands were scored for presence (1) or absence (0), and only fragments that were reproducible in at least three reactions were included for analyses, only polymorphic bands were considered

Band matching similarity coefficients (S) between isolates were calculated using the formula S = (2a / (a + b) + (a + c) where **b** and **c** are the number of fragments amplified in isolates **b** and **c**, respectively, and **a** is the number of bands shared by the two individuals (Nei and Li, 1979). Similarity coefficients were converted to genetic distance using the equation; (D = 1 – S) Using GenAlEx software (Peakall and Smouse, 2001). Then the genetic distance matrix was used to perform the following population genetic analysis:

i. Principal co-ordinate analysis (PCoA), the method for dominant data by Huff and colleagues (Huff et al., 1993) as implemented by GenAlEx (Peakall and Smouse, 2001) was followed, where the distance equals $D = [1 - 2n_{xy}/2n]$ with $2n_{xy}$ the number of shared bands between two individuals and 2n the total number of banding positions.

ii. The analysis of molecular variance (AMOVA) was performed by the previous software to partition the total genetic variation among and within targeted populations. The test of significance for the AMOVA was carried out on 99 permutations of the data.

Finally; distance tree (dendrogram) were Constructed using clustering with the Unweighted Pair Group Method with Arithmatic Mean (UPGMA). As implemented by SPSS version 17.0 software.

3.8. Soil analysis

Representative samples of the soil from each studied location were taken and analyzed via the NCAR Soil Analysis Laboratory in order to determine its major content and characteristics. Table 3.5 showed the tested parameters and the method applied.

Table 3.5
Investigated parameters and method in soil analysis performed via (NCAR) soil analysis laboratory.

Tested parameter	Method
PH	PH meter
EC	Conductivity meter
P	Olsen method using Ascorbic acid
K	Ammonium oxalate method
N	Kjeldahl digestion

CHAPTER FOUR
Results

4.1. Oil Yield

Variations in the oil yield obtained from J. phoenicea leaf and berry from three different geographical regions and two different parts are represented in Table 4.1.

Berry oil obtained from Petra and Dana sites characterized by comparatively higher yield also leaf and berry oils from the same locations gave the same yield irrespected the oils that taken from Wadi Rum location.

Table 4.1
Variations in the chemical composition of J. phoenicea essential Oils from three different geographical regions and two different parts

Site	Berries (% v/w)	Leaves (% v/w)
Petra (P)	2.4	0.59
Wadi rum (W)	1.0	1.26
Dana (D)	2.5	0.6

4.2. Chemical composition

Variations in the chemical composition of J. phoenicea essential oils from three different geographical regions and two different parts are represented in Table 4.2.

Fifty eight compounds were identified in both berry and leaf oil giving almost 99.5% of the oils composition·

Berries oil composed mainly of monoterpenoid hydrocarbons with average composition (83.75%) majored by α-pinene (70.03, 64.53, 78.41%), Myrcene (4.24, 3.17, 3.01%), Nonane (1.27, 1.29, 1.64 %), and Limonene (1.52, 1.42, 1.24 %) for W, P, D sample respectively. Oxygenated Monoterpene account for less composition (total average = 2.6%). Germacrene D represent the major hydrocarbones sesquiterpenes (3.46, 6.1,1.01 %) for W, P, D sample respectively, also it is the second abundant constituent of the berries oil composition. Epi-Cedrol present in low amount in Dana sample (.49%), however it represent the major oxygenated sesquiterpenes among Wadi rum and Petra samples respectively (3.1, 1.63 %).

The leaves oils obtained from Petra and Dana, were predominantly composed of monoterpene hydrocarbons (60.9, 64. 34%) with α-pinene as major constituent (41.7, 29.13%) followed by δ-3-Carene (11.9, 11.21%).

Nonane (1.2, 1.6 %) and Isopulegyl acetate (1.33, 1.2%), are the main oxygenated monoterpene while β-Funebrene is the major hydrocarbones sesquiterpenes (2.4, 2.63 %). Oxygenated sesquiterpenes was the second major important constituent of these samples (average = 29.42%) with epi-Cedrol as major compound followed by Allo-cedrol.

In contrary, Leaf oil obtained from Wadi rum was predominated by oxygenated sesquiterpenes (47.6 %). epi-Cedrol is the chief compound (42.7%) followed by Allo-cedrol (2.5%). monoterpene hydrocarbons represent the second major constituent (21.3%) and α-pinene (15.8%) was the second important compound followed by hydrocarbones sesquiterpenes majored by β-Funebrene (4.1%).

The analysis reveals the presence of one tentatively identified Oxygenated Monoterpene compound (Lilac alcohol??).

Table 4.2
Quantitative variation in the chemical composition of J. phoenicea essential oils obtained from three different geographical locations in Jordan and two part of tree.

#	Calc-RI	Lit-RI	Compound	%Content					
	Hydrocarbon Monoterpene (HM)			W. L	W.B	P.L	P.B	D.L	D.B
2	907	900	Nonane	1.1	1.27	1.2	1.29	0.94	1.64
3	924	918	2-methyl-4Heptanone	0.3	0.69	0.5	0.66	0.35	0.80
4	934	932	α-pinene	15.8	70.03	41.7	64.53	29.13	78.41
5	951	946	Camphene	0.1	0.69	1.0	0.58	0.72	0.84
6	974	969	Sabinene	0.1	0.68	0.1	0.48	0.05	0.91
7	980	974	β-pinene	0.3	2.19	0.8	1.84	0.68	2.22
8	989	988	Myrcene	0.4	4.24	1.5	3.17	1.31	3.01
9	1011	1008	δ-3-Carene	2.6	2.03	11.9	0.20	11.21	4.53
10	1026	1020	p-Cymene	0.1	0.05	0.3	0.05	0.05	0.05
11	1031	1024	Limonene	0.3	1.52	1.2	1.42	1.16	1.24
12	1059	1054	γ-Terpinene	0.1	0.05	0.3	0.30	0.05	0.05
	1088	1086	Terpinolene	0.1	0.64	0.4	0.54	0.69	0.41
			TOTAL	21.3	84.08	60.9	75.06	64.34	92.11
Oxygenated monoterpene (OM)									
13	1103	1095	Linalool	1.0	0.05	0.1	0.05	0.05	0.05
14	1147	1135	trans-Pinocarveol	0.5	0.05	0.1	0.05	0.05	0.05
15	1152	1141	Camphor	0.6	0.87	0.4	0.60	0.74	0.52
16	1165	1158	trans-Pinocamphone	0.1	0.05	0.3	0.05	0.41	0.05
17	1177	1166	p-Mentha-1,5-dien-8-ol	0.1	0.05	0.1	0.05	0.05	0.05
18	1185	1174	Terpinen-4-ol	0.5	0.29	0.1	0.05	0.45	0.05
19	1193	1181	Thuj-3-en-10-al	0.1	0.05	0.2	0.05	0.05	0.05
20	1200	1186	α-Terpineol	0.9	0.59	0.3	0.05	0.31	0.05
21	1230	1223	Citronellol	0.1	0.05	0.1	0.05	0.31	0.05
22	1248	NA	Lilac alcohol (??)	0.7	0.05	0.1	0.05	0.05	0.05
23	1251	1254	Linalool acetate	1.8	0.05	0.1	0.05	0.05	0.05

Continue Table 4.2

24	1273	1275	Isopulegyl acetate	1.9	0.05	1.2	0.05	1.33	0.05
25	1282	1287	trans-Linalool oxide acetate (Pyranoid)	1.5	0.05	0.1	0.05	0.42	0.05
26	1287	1287	Bornyl acetate	0.3	0.05	0.4	0.22	0.05	0.05
27	1337	1332	cis-Piperitol acetate	0.7	0.05	0.3	0.87	0.58	0.13
28	1349	1346	α-terpinyl acetate	2.2	0.43	0.8	0.89	1.49	0.32
			TOTAL	13	2.78	4.7	3.18	6.39	1.62
Hydrocarbon Sesquiterpene (HS)									
29	1391	1387	α-Duprezianene	0.4	0.05	0.1	0.56	0.05	0.05
30	1419	1413	β-Funebrene	4.1	0.23	2.4	0.28	2.63	0.30
31	1422	1417	(E)- Caryophyllene	0.9	1.42	0.4	2.87	1.03	0.49
32	1428	1419	β-Cedrene	1.0	0.05	0.6	0.05	0.56	0.05
33	1432	1434	γ-Elemene	0.1	0.05	0.1	0.42	0.05	0.05
34	1439	1429	cis-Thujopsene	0.8	0.05	0.4	0.05	0.43	0.05
35	1452	1448	cis-Muurola-3,5-diene	0.4	0.05	0.1	0.05	0.05	0.05
36	1459	1452	α-Humulene	0.7	0.78	0.3	0.94	0.85	0.22
37	1467	1465	cis-Muurola-4(14),5-Diene	0.3	0.05	0.1	0.05	0.05	0.05
38	1475	1475	trans-Cadina-1(6),4-Diene	0.6	0.20	0.1	0.05	0.65	0.05
39	1478	1478	γ-Muurolene	0.3	0.05	0.1	0.05	0.32	0.05
40	1484	1484	Germacrene D	1.1	3.46	0.5	6.61	1.73	1.01
41	1495	1493	trans-Muurola-4(14),5-diene	1.1	0.33	0.1	0.05	0.77	0.05
42	1563	1559	Germacrene B	0.4	0.71	0.1	5.32	1.32	0.49
43	1502	1500	α-Muurolene	0.3	0.26	0.1	0.05	0.33	0.05
44	1511	1505	α-Cuprenene	0.6	0.05	0.1	0.37	0.35	0.05
45	1515	1512	α-Alaskene	1.2	0.20	0.9	0.42	1.06	0.15
46	1521	1511	δ-Amorphene	1.8	0.74	0.5	0.59	2.31	0.11
47	1526	1522	δ-Cadinene	1.2	0.49	0.3	0.05	0.75	0.05
48	1538	1533	trans-Cadina-1,4-Diene	0.5	0.05	0.1	0.05	0.34	0.05
			TOTAL	17.8	9.27	7.4	18.88	15.6	33.42

Continue Table 4.2

Oxygenated Sesquiterpene (OS)									
49	**1553**	**1548**	**Elemol**	**0.1**	**0.05**	**0.1**	**0.31**	**0.05**	**0.05**
50	**1499**	**1493**	**epi-Cubebol**	**0.3**	**0.05**	**0.1**	**0.05**	**0.62**	**0.05**
51	**1581**	**1577**	**Spathulenol**	**0.0**	**0.05**	**0.1**	**0.24**	**0.36**	**0.05**
52	**1587**	**1582**	**Caryophyllene oxide**	**0.3**	**0.05**	**0.1**	**0.05**	**0.05**	**0.05**
53	**1594**	**1594**	**Carotol??**	**0.1**	**0.05**	**0.1**	**0.05**	**0.78**	**0.05**
54	**1602**	**1589**	**Allo-cedrol**	**2.5**	**0.05**	**1.2**	**0.05**	**1.51**	**0.05**
55	**1606**	**1608**	**β-Atlantol**	**0.5**	**0.05**	**0.3**	**0.05**	**0.05**	**0.05**
56	**1615**	**1618**	**epi-Cedrol**	**42.7**	**3.10**	**24.9**	**1.63**	**27.31**	**0.49**
57	**1633**	**1627**	**1-epi-Cubenol**	**1.0**	**0.27**	**0.2**	**0.05**	**0.86**	**0.05**
58	**1700**	**1700**	**Eudesm-7(11)-en-4ol**	**0.1**	**0.05**	**0.1**	**0.42**	**0.05**	**0.05**
			TOTAL	**47.6**	**3.77**	**27.2**	**2.9**	**31.64**	**0.94**

Key: Calc-RI: retention index, Lit-RI: Calculated Arithmetic (Kovats) retention index

W; Wadi rum **P;** Petra **D**; Dana **B**; berry oil **L**; leaf oil

4.2.1. Antibacterial activity of the essential oil

The Preliminary screening of the in vitro antibacterial activity of the essential oils from J. phoenicea leaves and berries was investigated using Hole-plate diffusion assay and MIC was determined. In this investigation four microorganisms were used as a model by measuring the inhibition zones in presence and absence of each essential oil. The variation in the antimicrobial activity of J. phoenicea essential oils from different geographical regions and different parts of tree are represented in table 4.3.

All tested oils (except the essential oils obtained from Wadi Rum) showed clear inhibition of the growth of all bacterial strains. The best antimicrobial activity was recorded against E.coli, where as the lowest was against P. aeruginosa. Leaf oil exhibited higher antibacterial activity than berry oil particularly against the four tested pathogens. The differences in the bioactivity achieved by leaves and berries portion were significant among most of the tested strains (Table 4.3).

Among leavs oil, samples from Wadi Rum exhibited the highest activity as compared with others. The highest activity was recorded against E. coli (mm= 20.0± .64) and MIC (4.0 µl/ml) and B. cereus (mm=18.83± 0.46) and MIC (5 µl/ml) whereas the lowest activity was shown to be against P. aeruginosa (mm=7.83± 0.39) which extracted from Dana sites.

Berry oil obtained from Petra location exhibited higher activity against all the tested bacteria with being E.coli the best (mm = 14.16 ± 0.39) and (MIC = 5 µl). On the other hand the oil obtained from Wadi rum site showed the lowest activity which was against S. aureus.

Table 4.3
Variation in the antimicrobial activity of J. phoenicea essential oils from three geographical locations in Jordan and among two part of tree

Locality	Plant part	Diameter of Inhibition Zone (mm) S. aureus	B. cereus	E. coli	P. aeruginosa
Wadi Rum	Leaf	16.0± 0.45^{ac}	18.83± 0.46^{ac}	20.0± .64^{ac}	13.0 ± 0.45^{ac}
	Berry	6.5± 0.31^{mc}	6.83 ± 0.39^{mc}	7.50± 0.32^{mc}	-
Petra	Leaf	14.5± 0.31^{ad}	16.33± 0.55^{ad}	16.83± .46^{ad}	11.0± 0.45^{ac}
	Berry	12.83± 0.46^{ad}	13.16 ± 0.39^{md}	14.16 ± 0.39^{md}	8.83± 0.46^{mc}
Dana	Leaf	13.0± 0.45^{ae}	13.0 ± 0.45^{ae}	15.0 ± .45^{ae}	11.0 ± 0.45^{ac}
	Berry	11.0± 0.45^{ae}	11.16 ± 0.46^{ae}	12.50 ± 0.32^{me}	7.83± 0.39^{me}
*ANOVA		p<0.05	p <0.05	p <0.05	p <0.05
Standard antibiotic control*:					
Chloramphenicol (30 µg\disk)		25.83 ± 0.39	30.6 ± .56	25.5 ± 0.32	18.83 ± .46
Tetracyclin (30µg\disk)		21.5± .52	20.83 ± .46	21.5 ± .52	7.50 ± 0.32
Ampicillin (10 µg\disk)		7.5± 0.32	7.83 ± 0.39	7.25 ± 0.32	0.00 ± 0.00
Tobromycin (10 µg\disk)		17.5± 0.32	20.5± 0.32	17.50 ± 0.32	20.83± .46
Gentamycin (10 µg\disk)		19.16± .46	19.83 ± 0.39	19.16 0.39	20.5± .32

a: Inhibition zone diameters are expressed as Means± standard error of mean (SE) for two samples of each essential oil analyzed individually in replicate. Means followed by different superscript letters in the same columns represent significant difference ($p < 0.05$). the strength of activity is presented as resistant (> 7mm), intermediate (>12mm) and susceptible (> 18mm).

Table 4.4
MIC value of essential oil from different population of Jordan expressed in µl /ml.

a: MIC is the minimum inhibitory concentration

locality	Plant part	MIC a			
		S. aureus	E. coli	B. ceruse	P. aeruginosa
Wadi Rum	Leaf	6.25	4.0	5.0	15.0
	Berry	-	-	-	-
Petra	Leaf	7.5	5.0	5.0	-
	Berry	15.0	10.0	10.0	-
	Leaf	10.0	5.0	6.25	-
Dana	Berry	-	-	17.5	-

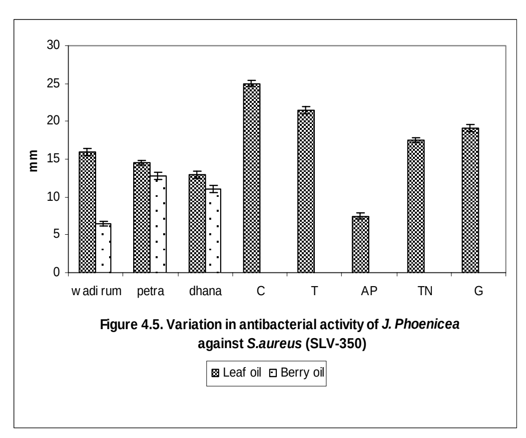


Figure 4.5. Variation in antibacterial activity of *J. Phoenicea* against *S.aureus* (SLV-350)

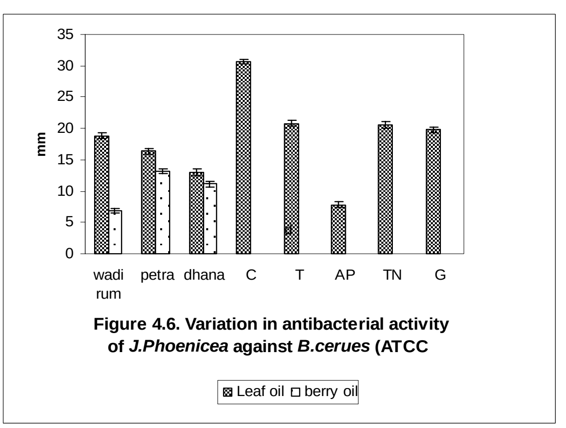


Figure 4.6. Variation in antibacterial activity of *J.Phoenicea* against *B.cerues* (ATCC

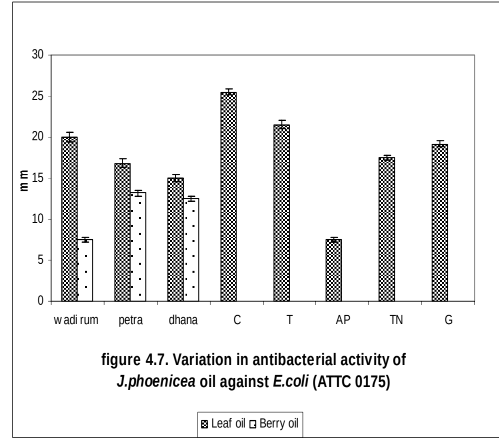


figure 4.7. Variation in antibacterial activity of *J.phoenicea* oil against *E.coli* (ATTC 0175)

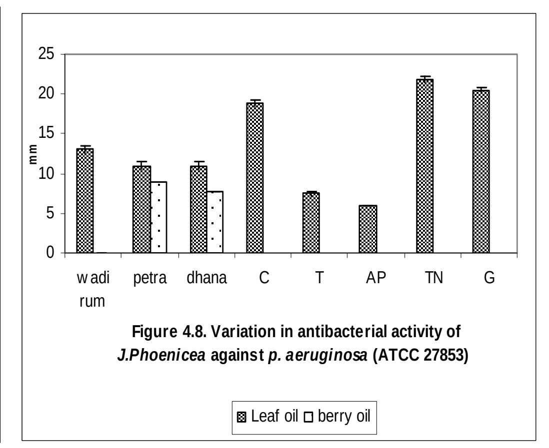


Figure 4.8. Variation in antibacterial activity of *J.Phoenicea* against *p. aeruginosa* (ATCC 27853)

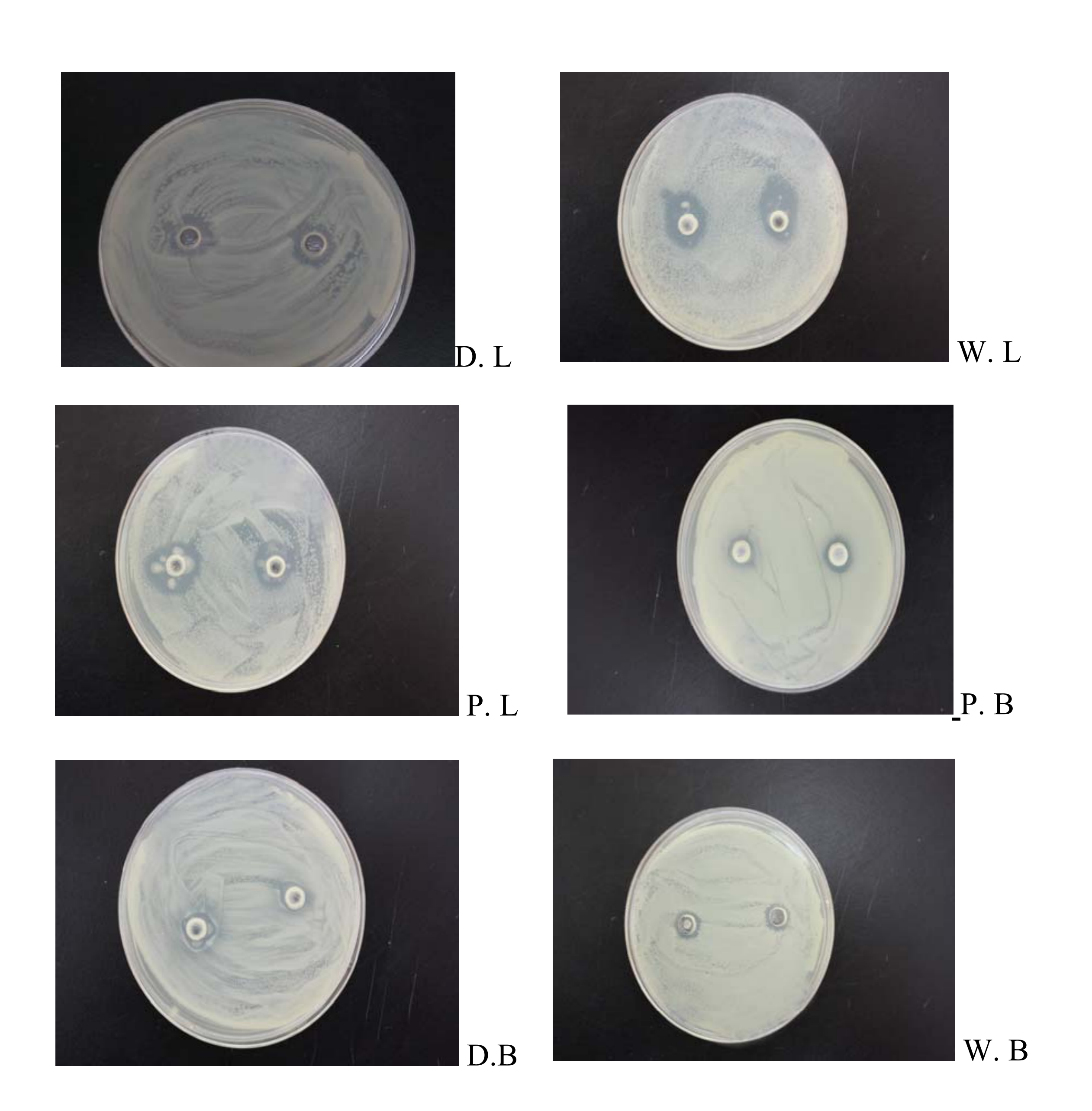


Figure 4.5. Hole plate diffusion test of J. phoenicea essential oils against B. ceruse ATCC 10875.

Key : W= Wadi rum , P= Petra , D= Dana
L = Leaf oil B= Berry oil

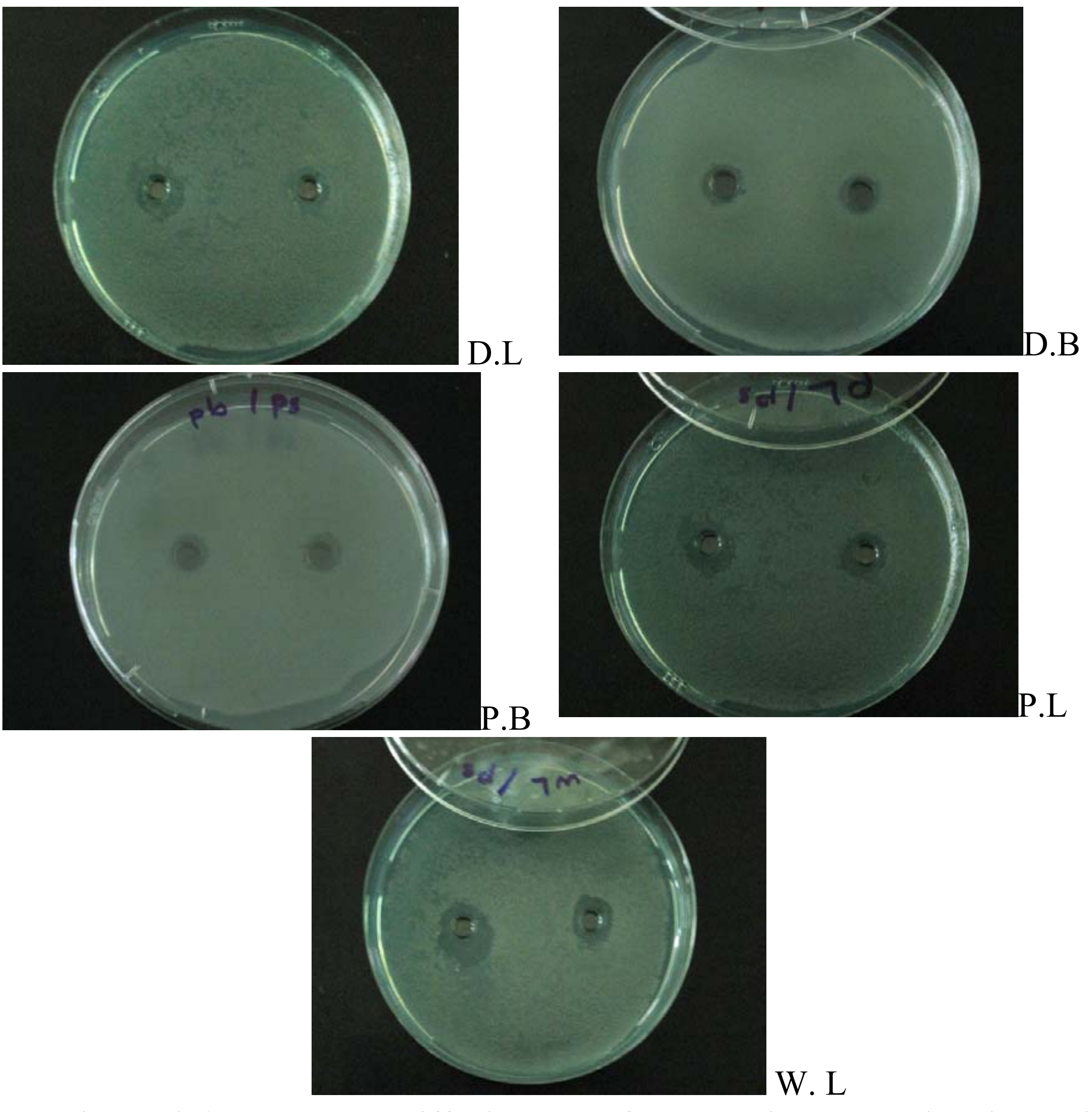


Figure 4.6. Hole plate diffusion test of J. phoenicea essential oils against P. aeruginosa ATCC 27853**.**

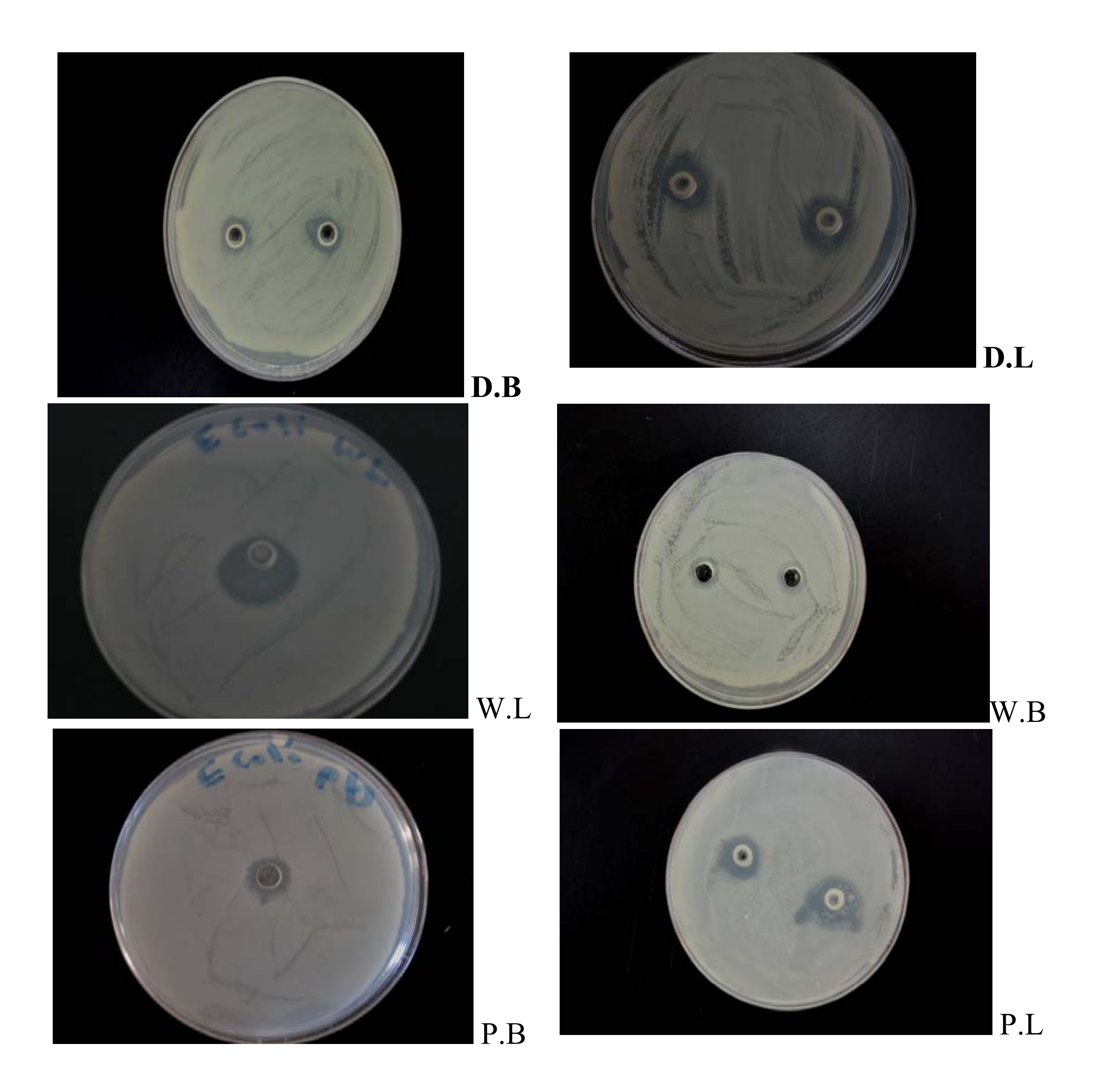


Figure 4.7. Hole plate diffusion test of J. phoenicea essential oils against E. coli ATCC 0175

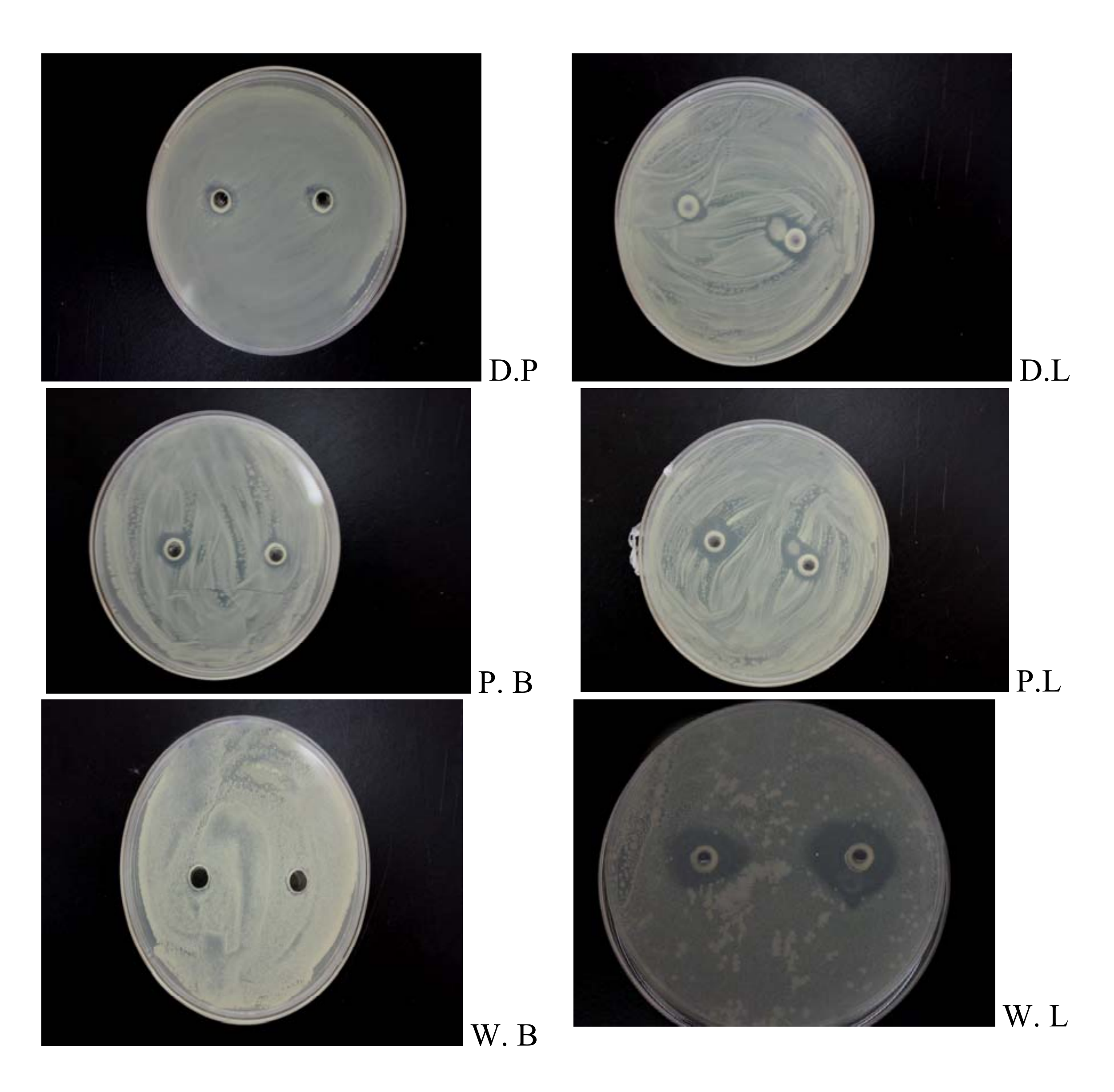
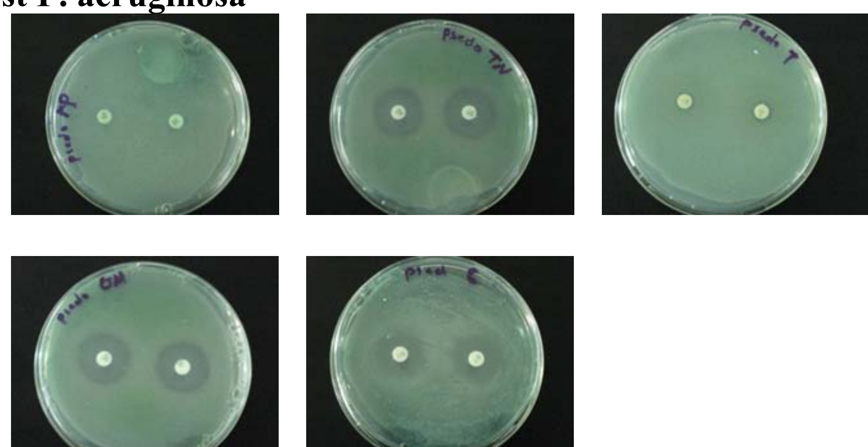
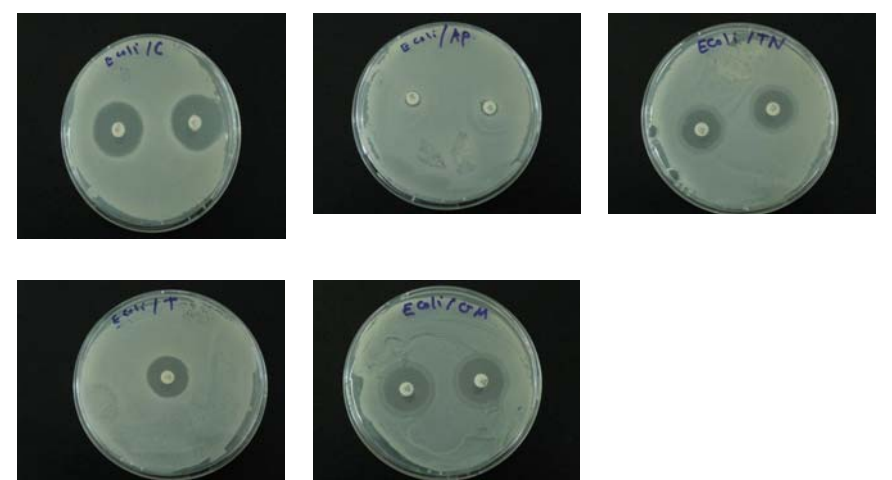


Figure 4.8. Hole plate diffusion test of J. phoenicea essential oils against S. aureus SLV-350.

I. Against P. aeruginosa

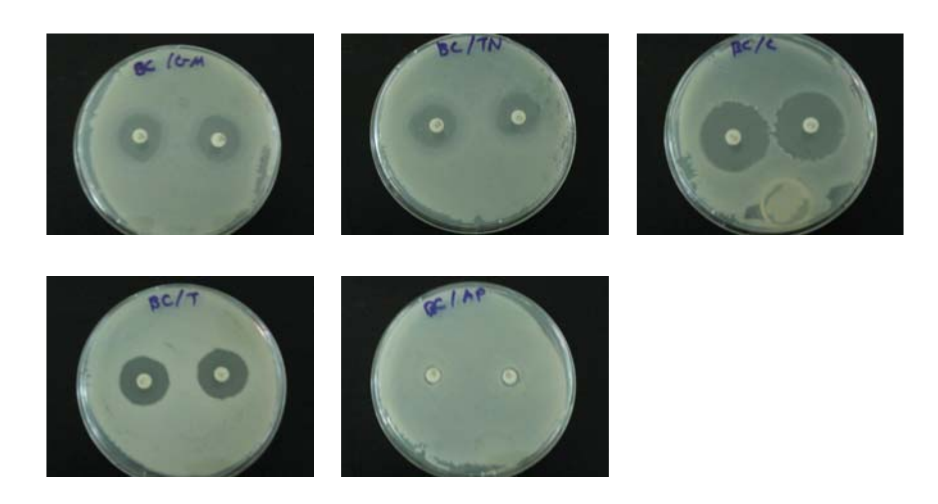


II. E.Coli



Continue figure 4.9

III. B. ceruse



IV. S. aureus

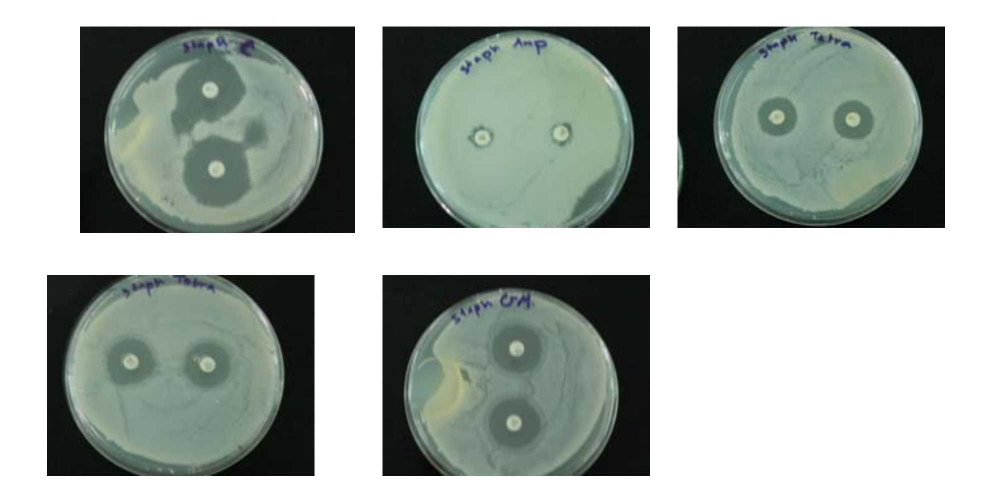


Figure 4.9. Hole plate diffusion test of Standard antibiotic disk : Chloramphenicol (30 µg\disk) Tetracyclin (30 µg\disk) Ampicillin(10 µg\disk), Tobromycin (10 µg\disk) and Gentamycin (10 µg\disk)

4.3. Genetic diversity

The Analysis of Molecular Variance- AMOVA combined to Principal co-ordinate (PCoA) and UPGMA analysis were performed for describing diversity status of the studied J. phoenicea populations

The information obtained by the analysis of the banding pattern is summarized in **(**Table 4.5**).** Three selected ISSR primers yielded 35

reproducible amplification products in the range 100 – 1200 bp. Figure 4.11 – 4.13, and the genetic distances between the five populations, as estimated by Nei's and lie, (1979), was Calculated and represented in Appendix 1.

Table 4.5
Descriptive Statistics of Three selected ISSR primers

Issr	Total bands	No. of polymorphic bands	No. of monomorphic bands	%of polymorphic (P %) bands
ISSR(AC)8–1	12	12	0	100 %
ISSR(AC)8–4	13	12	1	92.3%
ISSR(AT)8–2	10	8	2	80 %

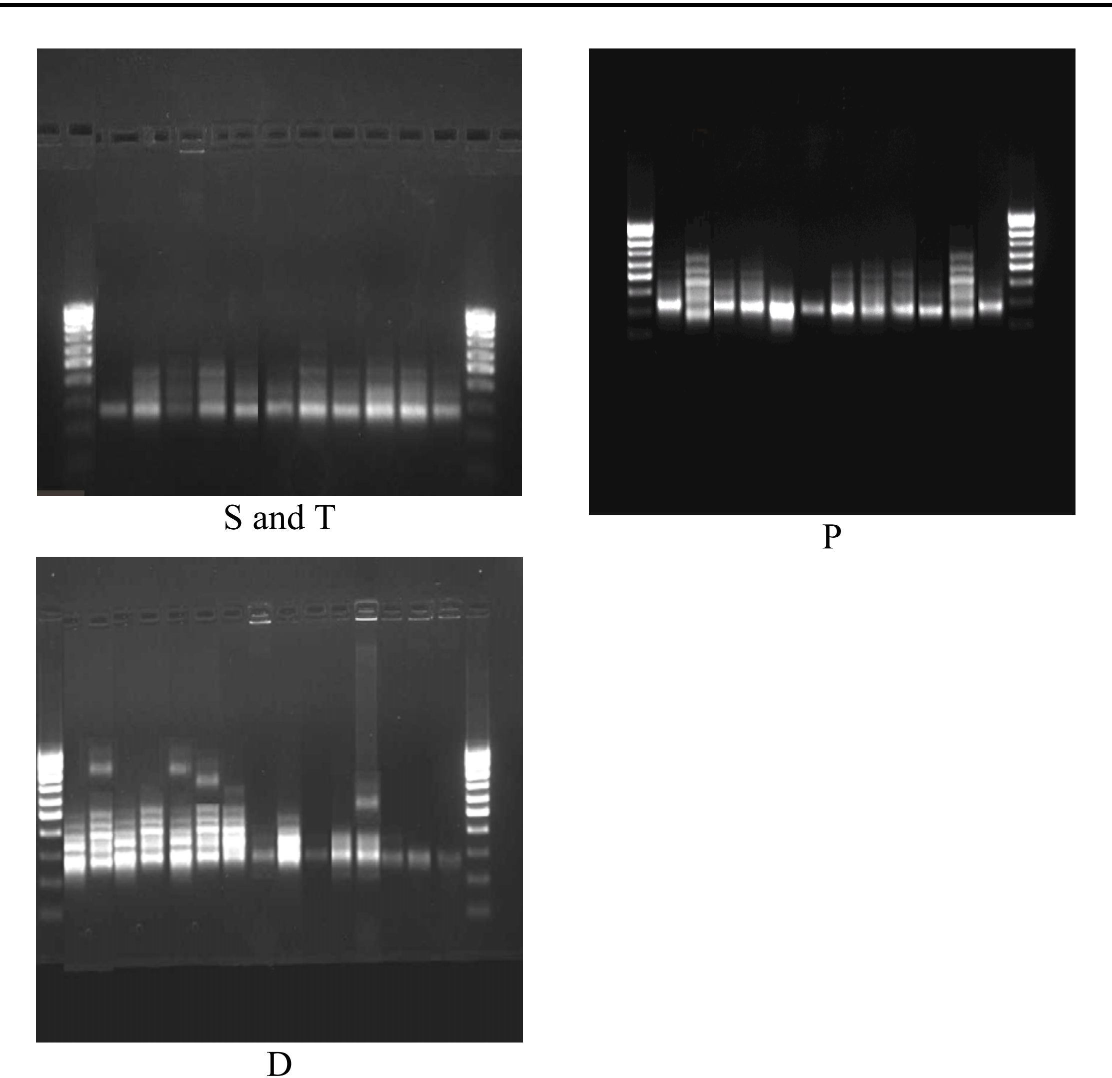


Figure 4.11. Amplification profiles of five populations Of J. phoenicea from Jordan with the primer ISSR (AC)8–1

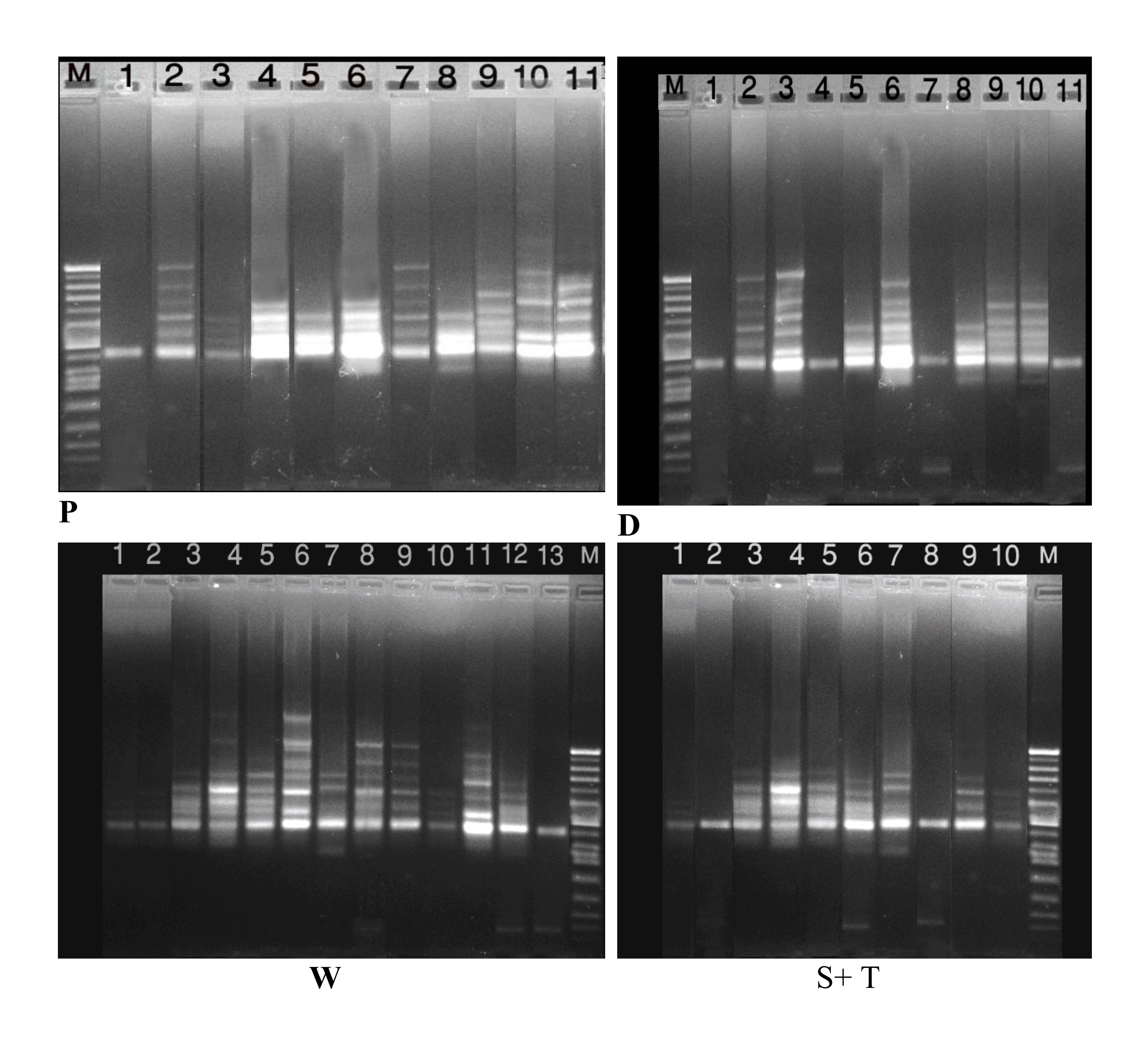


Figure 4.12. Amplification profiles of five pop. Of J.phoenicea trees from Jordan with the primer ISSR (AC) 8–4

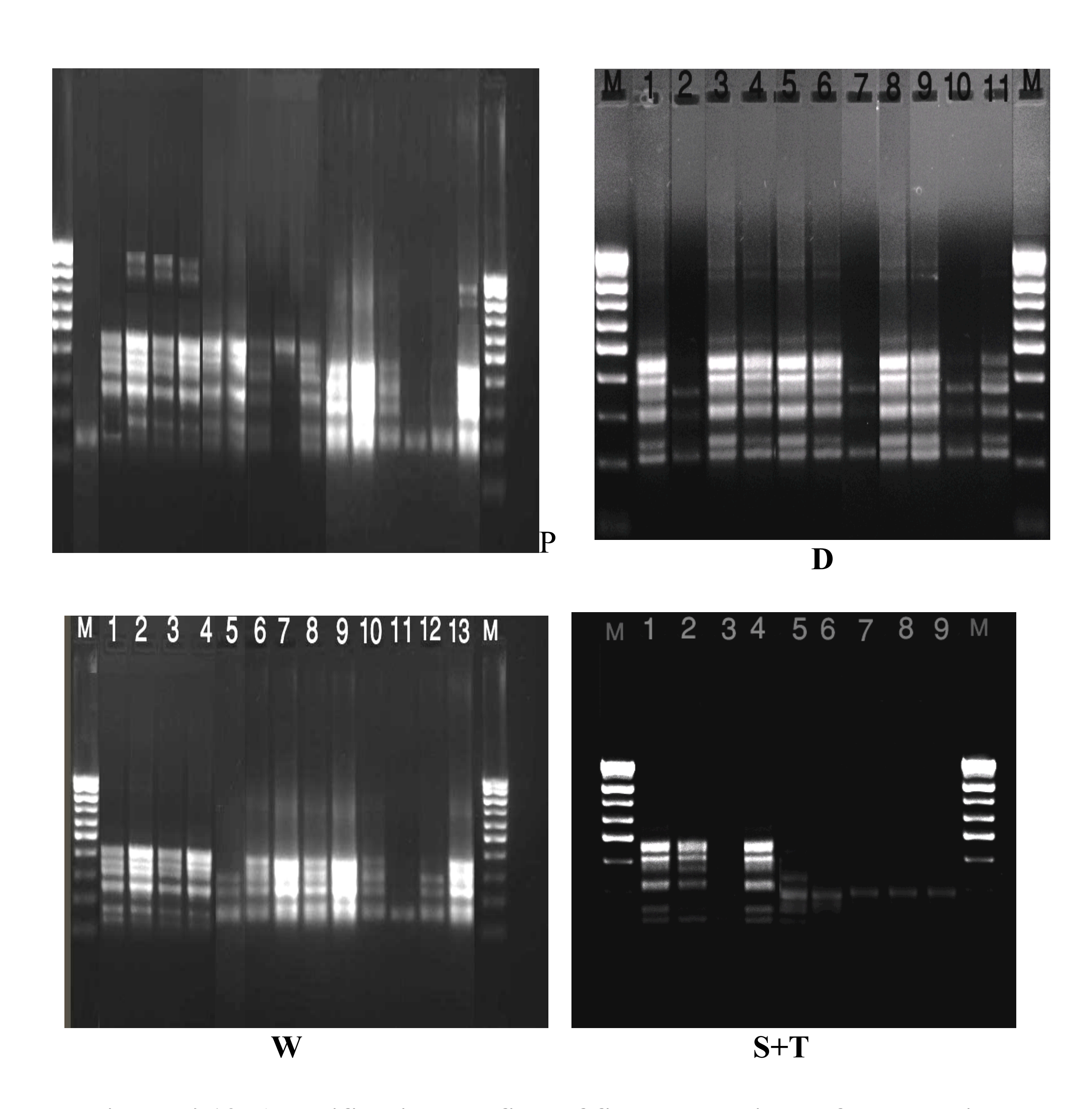


Figure 4.13. Amplification profiles of five populations of J. phoenicea from Jordan with the primer ISSR (AT) 8–2 .

4.3.1. AMOVA - Analysis of Molecular Variance

AMOVA was performing to calculate the proportion of the total diversity that is partitioned among populations (Φst) (Wright, 1951).

The total amount of genetic variation detected has been partitioned into its components due to the subdivision between populations and between individual within populations. The populations were divided into three geographical regions:

(i) Petra with 3 populations: Little Petra (P), AL- shoubak (S) and AL-tybeh (T)

(ii) Dana (D), one population.

(iii) Wadi rum (W), one population.

The results of AMOVA (Table 4.6 and figure 4.14) show that a small but significant amount of genetic variation (0.467% of the total) is due to differences among populations and that larger, significant amount (5.334% of the total) is due to differences among individuals within the same populations.

The proportion of genetic variation contributed by the differences between populations (Φst) is 0.081, thus leaving 92 %of the total genetic variation harboured within the populations analysed.

Table 4.6
Analysis of molecular variance (AMOVA) based on 35 ISSR markers for the five populations of Juniperus phoenicea from S. Jordan

Source of variation	d.f	Variance component	Est. Var. %	p
Among populations	4	9.690	0.467	0.020
Within populations	43	5.334	5.334	0.020
Total	47		5.801	0.020

P-value: Probability of null distribution.

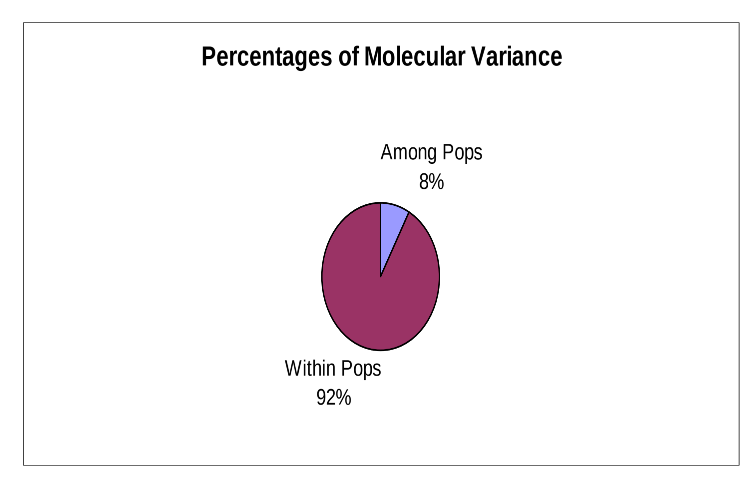


Figure 4.14. Analysis of molecular variance (AMOVA) based on 35 ISSR bands for the five populations of Juniperus phoenicea from S. Jordan
Regions analyzed are Petra with three populations, 'Dana 'and 'Wadi rum' with one population each.
(Φst) = 0.081 Probability, P(rand>=data), is based on 99 permutation across the full data set. (Φst) = AP / (WP + AP) = AP / TOT
Key: AP = Est. Var. Among Pops, WP = Est. Var. Within Pops

4.3.2. Multivariate analysis

A multivariate ordering (PCoA), based on pairwise genetic distances between the five studied populations and the individuals of all the five populations studied, was used to draw a distinction between the levels of inter and intra-population genetic structuring. (PCoA) gave the results shown in Figure 4.15 and 4.16. Each tree is plotted according to the first two principal co-ordinates.

The percentage of variation between populations explained by the first two co-ordinates amount for 100% of the variance between studied populations (Appendix 2). Where as the first two co-ordinates accounted for 41.11%, 21.07% of the total variance among individuals within the five populations (Appendix 3).

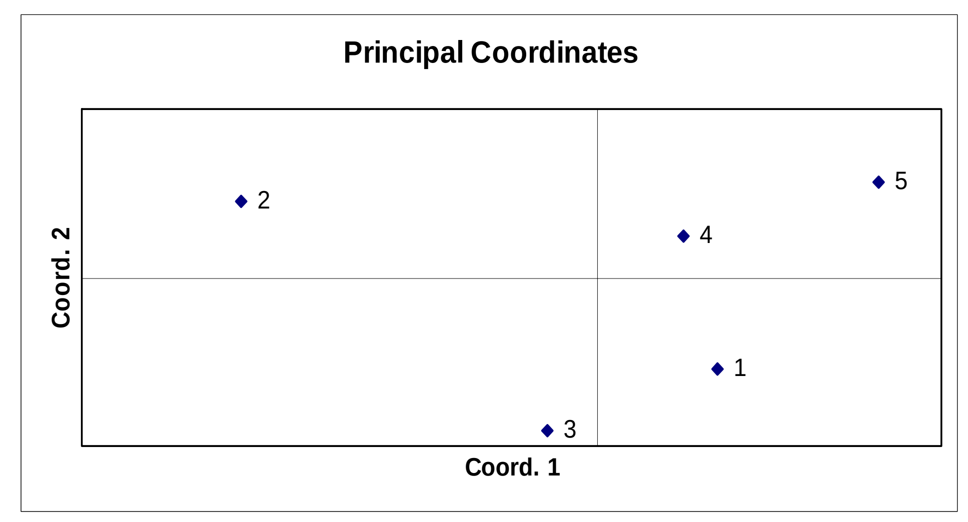


Figure 4.14. Principal co-ordinate analysis based on the multilocus genotype for the five populations studied. Each no. represents a single population as indicated (1: Dana, 2: Wadi rum, 3: Petra, 4: shoubak, 5: tybeh)

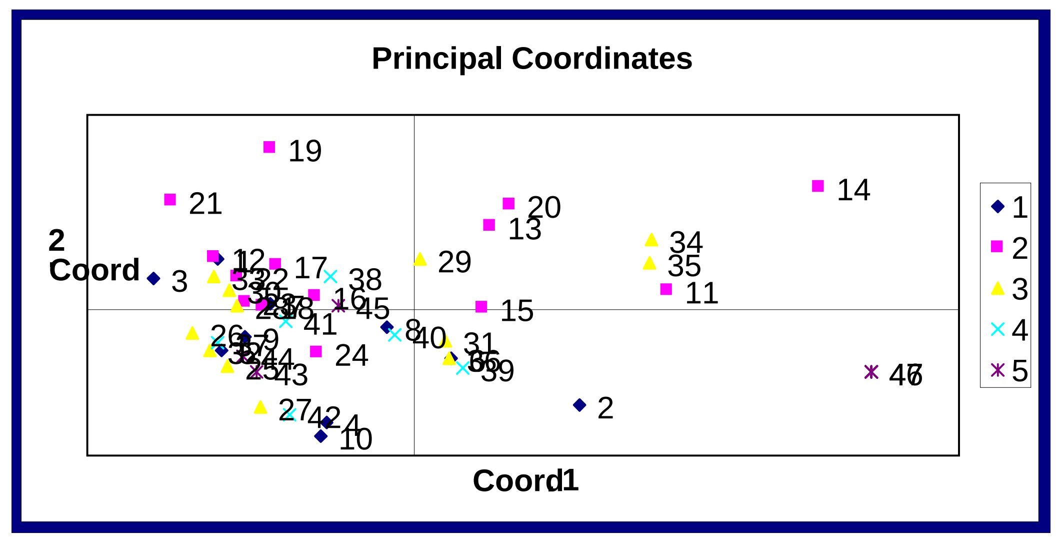


Figure 4.15. Principal co-ordinate analysis based on the multilocus genotype for the individuals of all the five populations studied Each symbol represents a single population tree as indicated (1 : Dana, 2 : wadi rum, 3: petra, 4: shoubak, 5: taybeh)

4.3.3. Distance Tree

The obtained distanced tree (The dendrogram Figure 4.17) showed that the five studied populations are grouped into three main clusters .The first cluster grouped the three populations from P, S and T locations while The

second cluster included population from P and D , and the third one linked Dana population together with Wadi rum.

The sub clusters under each main one enabled us to observe the genetic variation between individuals from each population and their relation with each other was detected.

4.4. Results of Soil Analysis

Table 4.7
Geographical variations in (PH, EC, P, K, and N) content in soils obtained from five location of J. Phoenicia (S. Jordan), and their Grouping

Location	PH	EC (Ds/m)	P (ppm)	K (ppm)	N (%)
Wadi Rum	7.9[a]	.36[a]	2.15[a]	33.7[a]	.04[a]
Dana	8[c]	1.46[c]	42.55[c]	266.1[d]	.23[c]
Petra	7.8[a]	6.22[e]	16.64[d]	331.1[e]	.07[t]
Al-tybeh	8[a]	.97[t]	7.65[t]	238.2[c]	.07[t]
Al- shoubak	7.8[a]	3.93[t]	99.87	226.4[t]	.28[d]

Rescaled Distance Cluster Combine

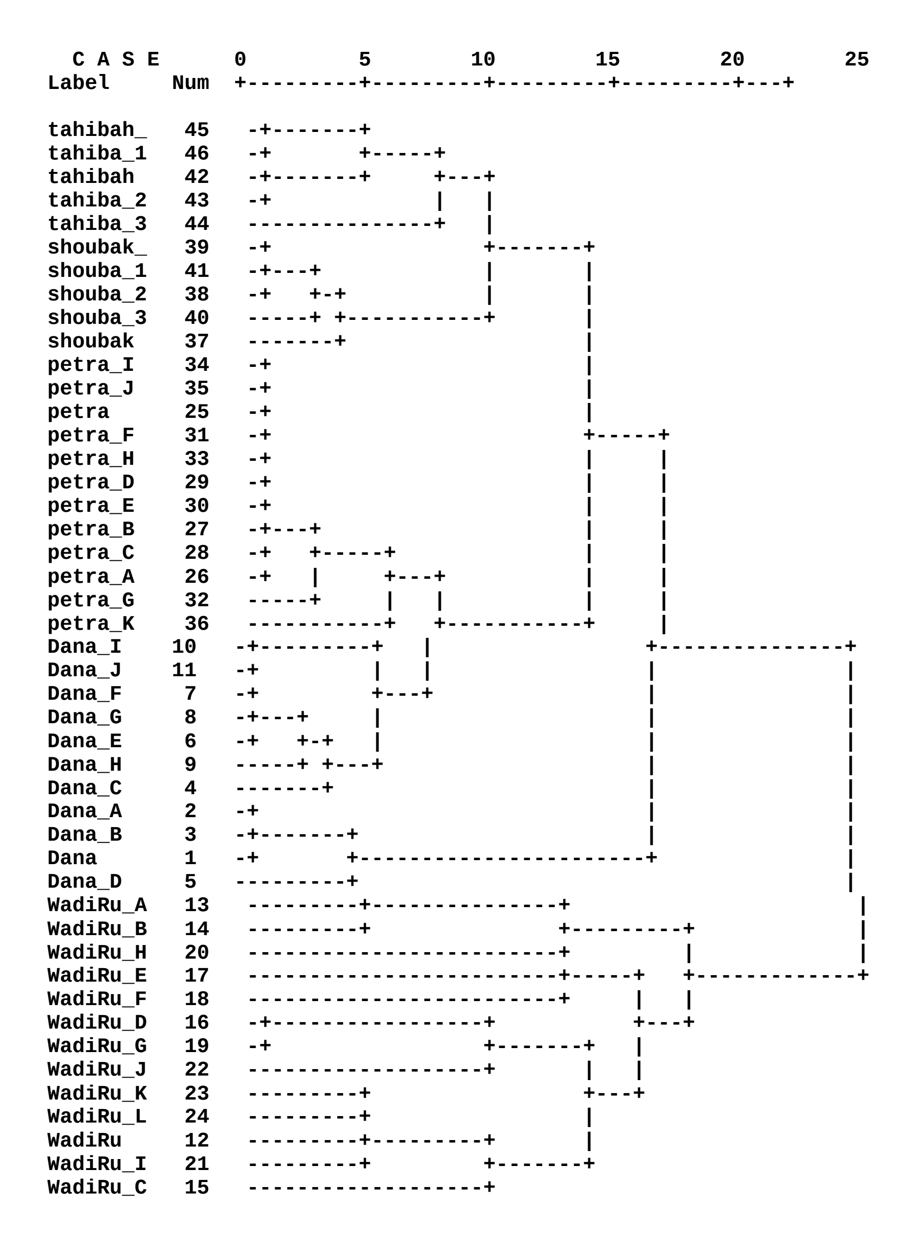


Figure 4.17. Dendrogram using Average Linkage (Between Groups)

CHAPTER FIVE
Discussion and Counclusion

This work forms a preliminary step to shed light on the biodiversity status among and within different populations of J. phoenicea from Jordan. The results of the anti bacterial activity, genetic and chemical analysis show a correlation with each other.

5.1. Genetic Variation among and within five natural population of J.phoenicea

Five populations of J. phoenicea from S.Jordan were analyzed by three polymorphic ISSR primers yielding 35 scorable bands. This number is low as compared with that of similar studies (based on ISSR) in other plant species. However it gives small window on genetic diversity study as preliminary study. Meloni et al. (2006) utilized three polymorphic ISSR primers yielding only 45 scorable bands to estimate genetic diversity of five Mediterranean populations of J. phoenicea and stated that this low number of polymorphic ISSR bands is useful to evaluate genetic diversity status of J. phoenicea as preliminary phase study.

The result of the PCA and UPGMA (Figure 4.16 and 4.17 respectively) together showed that J. phoenicea populations from Al-shobak, AL-Taybeh and Petra sites are placed together without any geographical arrangement. These populations are mixed together and with Dana population as well. This indicates that the current fragmentation resulted by human impacts and over grassing by cattle, sheep and goats may minimize the amount of genetic variation among the studied populations, which in turn reflects that these populations could came from the same origin (Douaihy et al., 2011) and did not get enough time to have large amount of genetic variation. This result is in agreement with the limited amount of genetic variation between populations predicted by the AMOVA (0.47 %).

AMOVA showed that individuals from different populations display lower genetic distance than trees within the same population. Previously Meloni and his group, (2005) record similar results in which the amount of genetic variation among five populations of J. phoenicea was 7% and the rest amount (93%) was harbored within the same population. One possible explanation for that is to be at least due to distribution range of J. phoenicea which considered an important factor that influencing the distribution of genetic diversity within a species (Meloni et al., 2006; Douaihy et al., 2011).

J. phoenicea forest along the southern heights (Al-Sharah mountains) has a populations scattered over a wide range this makes it more prone to

influence from the local environmental conditions. thus it display high values of population differentiation.

Population from high-elevation of Wadi rum appears to be somewhat in a separate group. It has larger population subdivisions than other studied populations as shown by UPGMA tree. Genotypes from this population appear to be distinct from other genotypes in the PCoA analysis with a quite tremendous amount of genetic variation appeared within it. This is likely due to geographical and ecological factors. For example, this population is geographically separated then makes it less subjected to fragmentation caused by the previously mentioned factors. Additionally, geographically isolated populations accumulate genetic differences as they adapt to different environmental conditions (Gupta et al., 2008). The soil analysis of this location was in parallel with the genetic diversity results (Table 4.7). It showed that this site have a soil with poor P, K and N contents, which in turn may affect the occurrence of unique genotype that was adapted to environmental stress.

On the other hand, the result of PCoA combined with UPGMA, shows some relation between Wadi Rum and Dana populations indicating that both populations likely came from the same unique origin.

Generally speaking there is agreement with a study performed by Douaihy et al. (2011) who characterized the genetic diversity among populations of J. excelsa from Lebanon and the eastern Mediterranean region. They found that the eastern high-elevation mount Lebanon were more strongly differentiated than populations from the rest of the range. They proposed that this would reflect a long period of isolation or possibly a different origin. They found that there is no significant correlation between geographic and genetic distances between the populations.

5.2. Essential oil of J. phoenicea

The chemical profile for samples of J. phoenicea collected from three locations in S. Jordan were studied and the chemical variation was assessed among them with respect to their geographical origin and part used in extraction. The obtained data give clear evidence that support the presence of variation among the studied samples. The recorded variations are easily recognized but mostly are quantitative. This is both for the yield and the composition of the oils.

5.2.1. Geographical variation in oil yield and composition

Oil yields of J. phoenicea from Jordan exhibited a variation with respect to their geographical origin. Samples from Petra and Dana gave the same yield and the differences were in Wadi rum sample (Table 4.1).

On the other hand geographical variation in essential oil yield of this plant are present compared with other geographical origin, the yield of leaf oil was higher than other plants studied in Portugal, Greece and Algeria in contrary it was lower than yield obtained from Spain, morocco, and Egypt (Table 5.1).

Table 5.1
Comparisons of yield for leaves essential oils of Juniperus phoenicea analyzed in other countries.

Region	yield %
Portugal (Robert et al., 1996)	0.41
Spain (Robert et al., 1996)	0.66
Greece (Robert et al., 1996)	0 .21
Egypt (Elsawi et al., 2007)	1.96
Morocco (Barrero et al., 2006)	0 .70
Morocco (Achak et al., 2009)	0.94
Morocco (Derwich et al., 2010)	1.62
Algeria (Mazari et al., 2010)	0.52

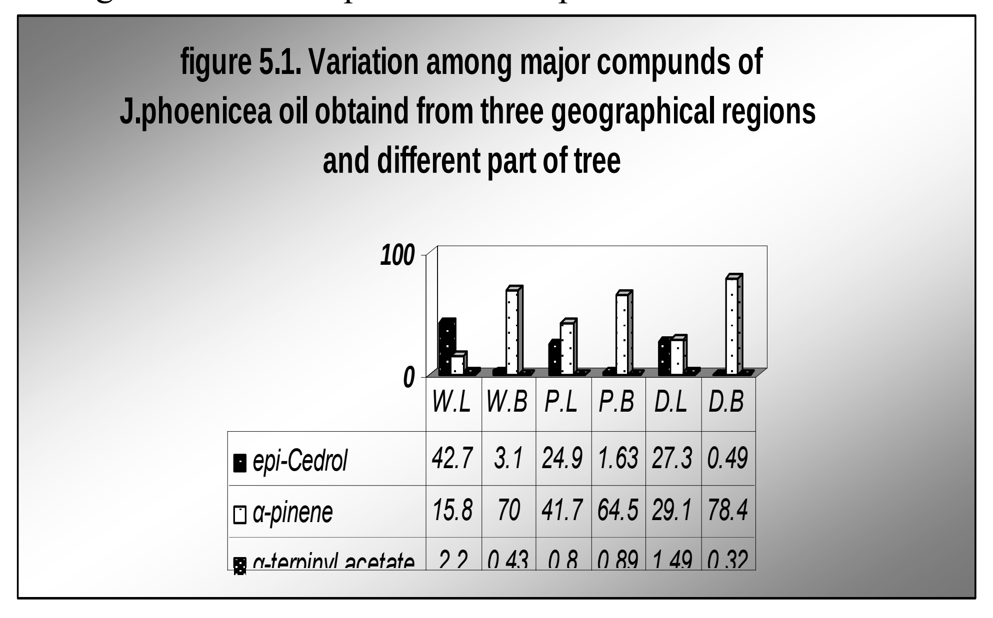
Most of the chemical variations among the studied location are observed between the major constituent while the minor one showed much less variation amount. This was particularly in leaf oil of Petra and Dana samples which demonstrated less variation into their chemical profile compared to Wadi rum sample (Table 4.2).

In general two profiles were seen in the terpenes composition of leaf oil (figure 5.1):

(i) Petra and Dana chemical profile; major content was Monoterpene Hydrocarbon (avrg = 63%) with α-pinene (29.13 and 41.7 %) followed by epi-Cedrol (24.9 and 27.31%), and δ-3-Carene (11.9 and 11.21%) for Petra and Dana respectively.

(ii) Wadi rum chemical profile; major content was oxygenated sesquiterpene (46.6%). epi-Cedrol was the chief compound (42.7%) and far less α-pinene (15.8%) content. followed by β-Funebrene (4.5%), Allo-cedrol (2.5%) and α-terpinyl acetate(2.2%).

The former profile agreed with most of recorded profile of leaf terpenes in different countries; Algeria, Morocco, Egypt, Spain, Portugal and Greece : Adams et al. (1996); Rezzi et al. (2001); Cavaleiro et al. (2001); El-Sawi et al. (2007); Adams et al. (2009); Derwich et al.(2010) and Mazari et al. (2010), In which that α-pinene and other pinane derivatives were the pre dominant composition in leaf oil of J. phoenicea with α-pinene as major constituent, cedrol and its derivatives were the second most important constituents and β-phellandrene was abundant component. In contrast to their find β-phellandrene was missing in Jordanian J. phoenicea samples.



Previous finding on other medicinal plant species have shown that the variation in their quantitative oil composition is attributed to the geographic direction (Rahimmalek et al., 2009; Hussain, 2009; Yavari et al., 2010). In this study the geographical differences in both yield and composition of the principal essential oil could be linked to the varied soil textures and content and possible adaptation response of different populations, resulting in different chemical products being formed, (Hussain, 2009; Yavari et al., 2010).

The revealed data from soil analysis showed that Wadi rum location had comparatively lower P, K and N content compared with soil in other location included in the study, accordingly this enhances richness in both yield and most of oil compounds of leaf oil from Wadi rum. This correlation may explained by the carbon/nutrient balance hypothesis (Bryant et al., 2008). Carbon/nutrient balance hypothesis includes that when nutrient availability in the soil is low, the low resource availability limits the growth of the plants

more than the photosynthesis, and plant allocates the extra carbon that cannot be used for growth to the production of carbon based secondary metabolites.

Other geographical factors such as; rainfall, temperature and altitude found to influence both the amount and composition of essential oils. (Rahimmalek et al., 2009; Yavari et al., 2010; Pirbalouti et al., 2011).

5.2.2. Effect of part used for extraction on oil yield and composition

The part used for extraction had an effect on the oil yield. Variation was noticed. The oil recovered from beery of J. phoenicea is relatively higher than oil recovered from its leaves. Irrespected to that obtained from Wadi rum location, also in contrary to El-sawi et al. (2007) study which found that berry oil recovered in lower yield compared to that obtained from leaf of J.phoenicea from Egypt.

The predominant composition in both berry and leaf oil characterized by a high content of α-pinene however berry oil demonstrated higher amount (Table 4.2). Myrcene and Germacrene D was the second important constituent in berry oil while it was Cedrol and δ-3-Carene in leaf oil.

The current data are in agreement with those obtained from previous studies performed to identify the major constituent of J.phoenicea in Italy, Egypt, and Tunisia (Angioni et al., 2003; El-Sawi et al., 2007 and Ennajar et al., 2009). All the mentioned studies compared the chemical composition of both leaf and berry oils and found that α-pinene, δ-3-carene, sabinene, myrcene, Cedrol and D-germacrene were among the most abundant constituent in both oil however quantitative and qualitative variation regard their chemical composition was present.

5.2.3. Genetic variation on chemical composition of J. phoenicea essential oils

The demonstrated chemical differences in this species can be most probably explained by the variability of the genetic factors as well. The current data reveal the presence of enough amounts of genetic diversity among the studied populations and the extreme was for Wadi rum population. This data are correlated with the existence of two chemo types among the studied population and again the extreme was for Wadi rum population. This suggests the presence of unique genes or alleles that might be responsible on increase the production of certain component of essential oil over the others, which in turn produce a variation in the chemical composition of the principal essential oil.

5.3. Antibacterial activities of J. phoenicea essential oils

In the last few years, there has been increasing interest in biologically active compounds isolated from plant species especially for medical and pharmacological purposes in addition to being ecologically safe compounds (Lee et al., 2005). The current study has investigate the antibacterial activity of J. phoenicea species obtained from leaves and berries against different pathogenic bacteria and evaluated the variability of this property according to geographical, environmental and genetic factors.

The determination of the inhibition zone by mean of the hole- plate diffusion method showed that J. phoenicea oil have potent antibacterial properties that significantly varied between the tested samples.

5.3.1. Variation Antibacterial activities J. phoenicea essential oils

This variation was significant among all tested samples regarding to their geographical origin and part of tree used in extraction as indicated in Table 4.3. The obtained data showed that leaf oil have more activity than berry oil. This data agreed with Angioni et al, (2003) and El-Sawi et al, (2007).

Leaf oil possess moderate to excellent inhibition of Gram-positive pathogens S. aureus and B. cereus (Table 4.3) mostly, samples belong to Wadi rum location. An excellent antimicrobial activity of the oil from this location could be attributed to the high of epi-cedrol content.

Over all, Gram-negative (P. aeruginosa) was the least sensitive of all the tested pathogens. However inhibition against this strain was obvious. Yet the strongest activity was for E. coli strain which recorded the lowest MIC value (Table 4.4). This result disagree with El-Sawi et al. (2007) as they found that J. phoenicea Leaf oil showed the lowest activity against E. coli and the strongest against B. subtilis. On the other hand this result agreed with Derwich et al., (2010) and Mazari et al., (2010) as they reported that E. coli was the most sensitive strain tested to the leaves oil of J. phoenicea. S. aureus were found to be sensitive to the same oil and P. aeruginosa was the least sensitive among all tested bacteria.

In contrast to leaf oil, the berry oil exhibited rather week to intermediate activity against most tested microorganisms, however both of them showed the best inhibition against E.coli, and the lower recorded against p. aeruginosa.

Several studies have reported that the Gram-negative bacteria, Pseudomonas, and in particular P. aeruginosa, appear to be least sensitive to the action of essential oils (Dorman et al., 2000; Glisic et al., 2007; Derwich et al., 2010; Mazari et al., 2010). There are several mechanism of resistancy used by different strains of bacteria including P. aeruginosa against essential oil which including enzymes such as β-lactamases which destroy the antibacterial

agent before it can have an effect. In addition, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect also bacteria may acquire several genes for a metabolic pathway which ultimately produces altered bacterial cell walls that no longer contain the binding site of the antimicrobial agent or bacteria may have mutations that limit access of antimicrobial agents to extracellular or intracellular target site. (Dorman et al., 2000; Tenover, 2006).

On another side, J.phoenicea oil essentially composed of Monoterpenes hydrocarbons (e. g., α-pinene, p-Cymene and Limonene) which frequently show antimicrobial properties that appear to be strong to moderate activity against Gram positive bacteria (Delaquis et al., 2001; Kim et al., 2003; Oyedeji and Afolayan, 2005). In fact, it is often recorded that Gram positive bacteria were more sensitive than Gram-negative to the essential oil in different medicinal plants. This is may be because of its cell wall which composed of single rather than multi layer structure which make it more sensitive to active compounds in essential oils.

In addition, the major compound of the investigated oil like, cedrol, δ-3-Carene and α-Terpinyl acetate have been known to exhibit antibacterial activity against the different bacterial strains among of which E. coli, S. aureus, B. cereus, and B.subtilis (Cosentino et al., 1999; Dorman et al., 2000; Glisic et al., 2007; Bourkhiss et al., 2007; Derwich et al., 2010). More over, enantiomers of α-pinene, β-pinene, limonene, linalool and other active terpenes have a strong antibacterial activity (Magiatis et al., 1999; Derwich et al., 2010) and can contribute to the overall antimicrobial effect of essential oils even at low concentration (Belletti et al., 2004).

many investigation showed that the main mechanism lies behind this antibacterial action of the major components of J. phoenicea oil that's C10 and C15 terpenes particularly with aromatic rings and phenolic hydroxyl groups as well as alcohols, aldehydes and esters are able to form hydrogen bonds with active sites of the target proteins or directly produce a disruption at the phospholipid bilayer membrane. These processes include the inhibition of electron transport, protein translocation, phosphorylation steps and other enzyme-dependent reactions (Knobloch et al., 1989; Cowan et al., 1999; Dorman et al., 2000; Belletti et al., 2004).

Over all, Variation in antimicrobial activity according to geographical origin is not easy to be explaining because several factors known to be co act within it. Intensive research has been conducted to explain this variation in antibacterial activity (Karousou et al., 2005; Yavari et al., 2010, and Pirbalouti et al., 2011). These studies have proved that such variation in antibacterial

activity linked directly with the respective composition of essential oil which in turn affected by several physiological, genetic, and environmental factors.

5.3.2. Comparison between J. phoenicea essential oil activities with some commercial antibiotics

Oil with the largest inhibition zones in this study, were compared with several antibiotics (Table 4.3). It can be seen that the oil showed a wide spectrum of inhibition. This oil showed the best inhibition against E.coli ATCC 0175 more than Ampicillin, Tobromycin and Gentamycin.

In case of P. aeruginosa (ATCC 27853), Tetracyclin and Ampicillin antibiotics showed no inhibiting effects, but the tested essential oil had stronger inhibition effects. Further it showed a stronger inhibition effect compared with Ampicillin against other tested strains. Similar result was recorded by Glisic et al. (2007) as they compared the inhibition effect of different fraction of juniper oil with some commercial drugs and found that α-pinene rich fraction showed a stronger inhibition effect against wide spectrum of bacteria over many antibiotics among of which Ampicillin.

The present data implies that essential oil or may be a fraction of this oil could be used as good conservation agents, against some food born pathogens but additional investigations need to be performed in order to confirm the safety of these concentrations (MIC) for human consumption.

5.4. Infra specific variation of J. phoenicea from Jordan

(i) Morphological evidence

In this investigation two distinct female cones (berry) morphology were found (Figure 5.2 – 5.5); the first one characterized by round shape (Globose) berry and was the predominant in all population exhibit that for Wadi rum population (Figure 5.2 and 5.3). The second is characterized by turbinate (Obovoid) berry shape was found in Wadi rum and rarly seen in Dana population (Figure 5.3 and 5.4). Previously, Gaussen, (1968), Adams, (2002), and Farjon, (2005) recorded two infraspecific variance or taxon in J. phoenicea: var. phoenicea with round shape female cone (berries) and var. turbinate with turbinate (= elongated) shape female cone.

This implies that both taxa of J. phoenicea are present in Jordan. The former may belong to var. phoenicea found in Petra, Dana, Al-Shoubak. While the latter belong to var. turbinate and found in Wadi Rum and Dana locations.

(ii) Leaf terpenoids evidence

A number of previouse studies have engaged in the taxonomy and characterization of Infraspecific variability of J. phoenicea made selection of Leaf terpenes composition with important amount of α-pinene, α-terpinyl acetate and b-phellandrene (Adams et al., 1996; Rezzi et al., 2001; Adams et al.,2009). These studies have differentiated two main types by the content of α-pinene, α-terpinyl acetate and β-phellandrene. The first is J. phoenicea var. phoenicea whose oil has higher quantities of α-pinene and lower of β - phellandrene. While the second is var. turbinate with two chemical types:

(i) High α-pinene, low β-phellandrene, low α-terpinyl acetate and (ii) low α-pinene, high β-phellandrene, high α-terpinyl acetate .

In comarasion, the obtaind data from the chemical analysis for leaf terpenes composition (section 4.2) indicate the presence of two profiles in J. phoenicea native to Jordan (Figure 5.1) :

(i) Petra and Dana chemical profile (only round berries included); major content is Monoterpene Hydrocarbon (avrg = 63%) with α-pinene (29.13 41.7 %) followed by epi-Cedrol (24.9 and 27.31%), and δ-3-Carene (11.9 and 11.21%) is the major constituent for Petra and Dana respectively.

(ii) Wadi rum chemical profile; major content is oxygenated sesquiterpene (46.6%) epi-Cedrol was the chief (42.7%) and far less α-pinene (15.8%) content followed by β-Funebrene (4.5%), Allo-cedrol (2.5%) and α-terpinyl acetate(2.2%).

It is obviously seen that chemical profile of those samples obtaind from Petra and Dana locations agree with that chemical profile previously recorded for J. phoenicea var. phoenicea whose oil has higher quantities of α-pinene and low amount of α-terpinyl acetate as mentioned above.

In comarasion, the chemical composition of Wadi rum population agreed with second types of J. phoenicea sup.sp. Turbinate. Which charecterised by low α-pinene amount and and relativly high α-terpinyl acetate as described previously by Rezzi et al. (2001).

(iii) Genetic variation evidence

The ISSR analysis reviled the presence of distinct genotype in Wadi rum J. phoenicea population which differs from those obtained from the other populations. Individuals from this population appear in separate group as shown by UPGMA tree. In addition, the result of PCoA combined with UPGMA, showed some relation between Wadi Rum and Dana populations indicating that J. phoenicea populations on both locations likely share some Infra specific type charechterised by exhibiting a turbinate feamale cones.

Although a combined analysis of morphological observation, chemical and genetic data does partly support the presence of some Infra specific

variation within J. phoenicea in Jordan, this result is tentative because the data obtained from dominant marker like (ISSR) is still not enough to unrevealing the situation. Further investigation by using more robust genetic markers like microsatellites or SNPs and a true phylogeographical analysis based on cpDNA are required to provide much clear identification (Meloni et al., 2005; Mao et al., 2010).



Figure 5.2. Round berry shape, Petra Site **Figure 5.3. Round berry shape, Dana Site**





Figure 5.4. Turbinate berry, Wadi Rum site **Figure 5.5. Turbinate berry, Dana site**

The research work presented in this thesis was accomplished to investigate the genetic variation, the yield, chemical composition, and antibacterial activities of the essential oils of J. phoenicea from south Jordan. Efforts were also made to appraise the effect of part of tree, geographical and genetic variations on the yield, chemical composition and biological activities of the essential oils isolated.

The analysis of (35) ISSRs bands of populations belonging to five geographical region of J. phoenicea in S. Jordan had success in analyzing the distribution of genetic variability among and within the studied populations, showing that 92 % of the total genetic variation is still harbored within populations. So this study has given important clues in understanding genotypic relationship of J. phoenicea populations in the kingdom. This may further assist in developing corrective conservation strategies to help halting J. phoenicea decline in Jordan.

The analysis successfully revealed the identification of ~99.5% of the total volatile products for J. phoenicea essential oil; this is both in beery and leaf oil. Monoterpene Hydrocarbon was mostly the major content in all tested samples except that for Wadi rum leaf oils which exhibited far more content of oxygenated sesquiterpene (46.6%) and its offered the Maximum essential oil yields among the tested leaf oils.

The analyzed essential oils showed a potent in vitro antibacterial activity against a panel of gram positive and negative bacteria, reveled that leaf oil obtained from Wadi rum location had stronger antimicrobial activity, as evident from its larger inhibition zones (13.0- 20.0 mm) and smaller MIC values (4- 15 μl/ml). over all, the antibacterial activity of the essential oil of J. phoenicea could be attributed to its content of epi-cederol, α-pinene and α-Terpinyl acetate, also the over all chemical constituents of this oil.

The obtained data showed that J. phoenicea oil could be used against both gram positive and negative bacteria. Further more it can be employed in place of some antibiotics against some food born pathogens but additional investigations need to be performed in order to confirm the suitable concentrations (MIC) for human consumption.

The present variations in the yield, chemical profile and biological activities of most the analyzed J. phoenicea essential oils, collected from different geographical regions of the country might have been due to the reason that environmental conditions such as soil type also the variation in genetic make up which can influence the regulation of the biosynthesis of essential oils.

As is evident from the presented genetic, chemical and morphological observation, this study suggests the presence of possible Infra specific variation within J.phoenicea from Jordan. The first type may belong to var. phoenicea and characterized by round shape berry which found in Petra, Dana and al- shobak. While the latter belong to var. turbinate, characterized by turbinate berry shape and found in Wadi Rum and rarely in Dana location. We suggest that Increase focusing and studying should be for J. phoenicea from Wadi rum region.

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Appendix (I)

Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea oil from different geographical locations and different part used for extraction (berry and leaf) against S. aureus

1.1. Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea leaf oil from different geographical locations against S. aureus

Source of variation	df	SS	MS	F	P-value
Among groups	2	13.5	6.75	9	0.015625
Within groups	6	4.5	0.75		
Total	8	18			

1.2. Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea berry oil from different geographical locations against S. aureus

Source of variation	df	SS	MS	F	P-value
Among groups	2	63.72222	31.86111	40.96429	0.000318
Within groups	6	4.666667	0.777778		
Total	8	68.38889			

1.3. Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea oil from different geographical locations and different part used for extraction (berry and leaf) against S. aureus

Source of variation	df	SS	MS	F	P-value	Geographical region
Among groups (Wadi rum)	1	135.375	135.375	216.6	0.000124	Wadi rum
Within groups	4	2.5	0.625			
Total	5	137.875				
Source of variation	df	SS	MS	F	P-value	petra
Among groups	1	4.166667	4.166667	6.25	0.066767	
Within groups	4	2.666667	0.666667			
Total	5	6.833333				
Source of variation	df	SS	MS	F	P-value	dana
Among groups	1	6	6	6	0.070484	
Within groups	4	4	1			
Total	5	10				

Appendix (II)

Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea oil from different geographical locations and different part used for extraction (berry and leaf) against E. coli

2.1. Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea leaf oil from different geographical locations against E. coli

Source of variation	df	SS	MS	F	P-value
Among groups	2	38.38889	19.19444	9.465753	0.013938
Within groups	6	12.16667	2.027778		
Total	8	50.55556			

2.2. Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea berry oil from different geographical locations against E. coli

Source of variation	df	SS	MS	F	P-value
Among groups	2	57.55556	28.77778	79.69231	4.77E-05
Within groups	6	2.166667	0.361111		
Total	8	59.72222			

2.3. Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea oil from different geographical locations and different part used for extraction (berry and leaf) against E. coli

Source of variation	df	SS	MS	F	P-value	Geographical region
Among groups	1	234.375	234.375	110.2941	0.000465	Wadi rum
Within groups	4	8.5	2.125			
Total	5	242.875				
Source of variation	df	SS	MS	F	P-value	petra
Among groups	1	20.16667	20.16667	24.2	0.007933	
Within groups	4	3.333333	0.833333			
Total	5	23.5				
Source of variation	df	SS	MS	F	P-value	dana
Among groups	1	9.375	9.375	15	0.017948	
Within groups	4	2.5	0.625			
Total	5	11.875				

Appendix (III)
Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea oil from different geographical locations and different part used for extraction (berry and leaf) against B. cereus

3.1. Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea oil from different geographical locations and different part used for extraction (berry and leaf) against B. cereus

Source of variation	df	SS	MS	F	P-value	Geographical region
Among groups	1	216	216	259.2	8.71E-05	Wadi rum
Within groups	4	3.333333	0.833333			
Total	5	219.3333				

Source of variation	df	SS	MS	F	P-value	
Among groups	1	15.04167	15.04167	10.31429	0.03254	petra
Within groups	4	5.833333	1.458333			
Total	5	20.875				

Source of variation	df	SS	MS	F	P-value	dana
Among groups	1	5.041667	5.041667	4.84	0.092653	
Within groups	4	4.166667	1.041667			
Total	5	9.208333				

3.2. Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea leaf oil from different geographical locations against B. cereus

Source of variation	df	SS	MS	F	P-value
Among groups	2	51.38889	25.69444	17.45283	0.003156
Within groups	6	8.833333	1.472222		
Total	8	60.22222			

3.3. Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea berry oil from different geographical locations against B. cereus

Source of variation	df	SS	MS	F	P-value
Among groups	2	62.88889	31.44444	41.92593	0.000298
Within groups	6	4.5	0.75		
Total	8	67.38889			

Appendix (IV)
Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea oil from different geographical locations and different part used for extraction (berry and leaf) against P. aeruginosa

4.1. Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea oil from different geographical locations and different part used for extraction (berry and leaf) against P. aeruginosa

Source of variation	df	SS	MS	F	P-value	Geographical region
Among groups	1	253.5	253.5	507	2.3E-05	Wadi rum
Within groups	4	2	0.5			
Total	5	255.5				

Source of variation	df	SS	MS	F	P-value	
Among groups	1	7.041667	7.041667	6.76	0.060048	petra
Within groups	4	4.166667	1.041667			
Total	5	11.20833				

Source of variation	df	SS	MS	F	P-value	
Among groups	1	15.04167	15.04167	19	0.012073	dana
Within groups	4	3.166667	0.791667			

4.2. Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea leaf oil from different geographical locations against P. aeruginosa

Source of variation	df	SS	MS	F	P-value
Among groups	2	8	4	4	0.078717
Within groups	6	6	1		
Total	8	14			

4.3. Percentage of variation and degree of freedom of the antimicrobial activity of J. phoenicea berry oil from different geographical locations against P. aeruginosa

Source of variation	df	SS	MS	F	P-value
Among groups	2	140.3889	70.19444	126.35	1.25E-05
Within groups	6	3.333333	0.555556		
Total	8	143.7222			

Appendix (V)
Genetic distance between five studied population of J.Phoenicea

Genetic distance between pop , Pairwise Population Matrix of Mean Pop Binary Genetic Distance

D	W	P	S	T	
10.378	12.500	10.067	9.860	11.557	D
12.033	11.476	11.614	14.031		W
9.636	10.000	11.881			P
10.000	10.771				S
10.476					T

Appendix (VI)
Percentage of variation between five populations of Juniperus phoenicea from S. Jordan based on 3 ISSR markers

Axis	1	2	3
%	82.17	17.83	0.00
Cum %	82.17	100.00	100.00

Appendix (VII)
Percentage of variation between individuals within five populations of Juniperus phoenicea from S. Jordan based on 3 ISSR markers

Axis	**1**	**2**	**3**
%	**41.11**	**21.07**	**14.71**
Cum %	**41.11**	**62.19**	**76.89**

Appendix (VIII)

LSD grouping for antimicrobial activity interaction between bacteria and leaf oil of wadi rum location and five commercial Antibiotics

Bacteria strain	treatment	Mean (mm)	Grouping
S.	J.phoenicea oil	14.5000	E
S. aureus	Chloramphenicol	25.8333	A
S. aureus	Tetracyclin	21.5000	B
S. aureus	Ampicillin	7.5000	G
S. aureus	Tobromycin	17.5000	D
S. aureus	Gentamycin	19.1667	C
Bacteria strain	**Treatment**	**Mean**	**Grouping**
B. cereus	J.phoenicea oil	18.83333	C
B. cereus	Chloramphenicol	30.6667	A
B. cereus	Tetracyclin	20.8333	B
B. cereus	Ampicillin	7.8333	E
B. cereus	Tobromycin	20.5000	B
B. cereus	Gentamycin	19.8333	B
Bacteria	**Treatment**	**Mean**	**Grouping**
E. coli	J.phoenicea oil	20.0000	C
E. coli	Chloramphenicol	25.5000	A
E. coli	Tetracyclin	21.5000	B
E. coli	Ampicillin	7.5000	G
E. coli	Tobromycin	17.5000	E
E. coli	Gentamycin	19.1667	D
Bacteria	**Treatment**	**Mean**	**Grouping**
P. aeruginosa	J.phoenicea oil	13.	D
P. aeruginosa	Chloramphenicol	18.8333	C
P. aeruginosa	Tetracyclin	7.5000	E
P. aeruginosa	Ampicillin	6.0000	F
P. aeruginosa	Tobromycin	21.8333	A
P. aeruginosa	Gentamycin	20.5000	B